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From: Lee, Jennifer
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1. Kubbutat et al., "Proteolytic cleavage of human p53 by calpain: A potential regulator of protein stability." *Molecular and Cellular Biol.* (1997), Vol. 17 (1): 460-468.
2. Zhang et al., "Inhibition of the growth of WI-38 fibroblasts by benzyloxycarbonyl- Leu-Leu-Tyr diazomethylketone: Evidence that cleavage of p53 by a calpain-like protease is necessary for G-1 to S-phase transition." *Molecular Biol. of the Cell* (1996), Vol. 7 (suppl): 27A
3. Gonen et al., "On the involvement of calpains in the degradation of the tumor suppressor protein p53." *FEBS Letters* (1997), Vol. 406 (1-2): 17-22.
4. Pariat et al., "Proteolysis by calpains: A possible contribution to degradation of p53" *Molecular and Cellular Biol.* (1997), Vol 17 (5): 2806-2815.
5. Shinohara et al., "Apoptosis induction resulting from proteasome inhibition." *Biochem. J.* (1996), Vol. 317 (2): 385-388.
6. Atencio et al., "Calpain inhibitor 1 activates p53-dependent apoptosis in tumor cell lines." *Cell Growth Differ.* (2000), Vol. 11 (5): 247-253.

Thanks,
Jennifer

Gai (Jennifer) Lee
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(FILE 'HOME' ENTERED AT 16:50:40 ON 22 JUN 2000)

FILE 'MEDLINE, CAPLUS, BIOSIS' ENTERED AT 16:51:09 ON 22 JUN 2000

L1	0 S REGULATING CELLULAR LEVELS OF P53
L2	54198 S P53
L3	3527 S L2 AND INHIBITOR
L4	34 S L3 AND CALPAIN
L5	2 S L4 AND (VIRAL VECTOR)

155

IDENTIFICATION OF A NOVEL CDC34 DEPENDENT UBIQUITIN-PROTEIN LIGASE IN YEAST. ((A. Banerjee and V. Chau)) Department of Pharmacology, Wayne State University School of Medicine, Detroit, MI 48201.

Ubiquitination targets regulatory proteins for selective degradation in eukaryotic cells. The yeast *S. cerevisiae* CDC34 gene encodes a ubiquitin-conjugating enzyme that is required for G₁/S phase transition in the cell cycle. We have isolated a dominant negative mutant of this enzyme (*J. Biol. Chem.* (1995) 270, 26209) and suggest that Cdc34^{DN} selectively sequesters a subset of Cdc34 binding proteins and/or substrates. Extragenic suppression of this allele has allowed us to isolate a novel ubiquitin-protein ligase (E3) in yeast encoded by a suppressor, *SUPF*. As expected of a Cdc34 binding protein, SupF can be co-immunoprecipitated in a complex with Cdc34. We have also been able to isolate a cellular complex that has Cdc34, SupF and Cdc4. In substrate ubiquitination, Cdc34 functions by forming a transient complex with ubiquitin and this bonded ubiquitin can subsequently be transferred to SupF *in vitro*.

(Supported by NIH grant GM47604)

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CELL ADHESION AND TYROSINE KINASES REGULATE G1/S PHASE TRANSITION AFFECTING THE RELATIVE AMOUNTS OF CYCLIN-DEPENDENT KINASES TO THEIR INHIBITORS. ((T. Kuzumaki, A. Matsuda, and K. Ishikawa)) Department of Biochemistry, Yamagata University School of Medicine, Yamagata 990-23, Japan.

Cell adhesion to substratum and activation of tyrosine kinases are essential for the progression of cell cycle through G₁ phase in mammalian cells. Cyclin-dependent kinase (CDK)-2, -4, -6, and their inhibitors, p27kip1 and p21cip1, are key regulators for the progression of G₁ phase. The kinetic studies of mouse Balb/c 3T3 fibroblasts showed that cell adhesion was essentially required in late G₁ phase, especially at the period of G₁/S transition. On the other hand, tyrosine kinase inhibitors, genistein and herbimycin A, also blocked the G₁/S transition most effectively when cells were exposed to the inhibitors at the period of G₁/S transition. The expressions of histone H2B and dihydrofolate reductase genes (S phase specific genes) were suppressed when cells were cultured without adhesion or exposed to the tyrosine kinase inhibitors. The amount of p27 was down-regulated in the G₁/S phase transition for cells to enter S phase and the amount of CDK2 was not changed throughout G₁ phase. However, when cells were cultured without adhesion or exposed to the tyrosine kinase inhibitors, CDK2 was decreased and the relative amount of p27 to CDK2 remains high level at the period of G₁/S phase transition. These results suggest that cell adhesion to substratum and tyrosine kinases play an important role in the G₁/S phase transition of mouse Balb/c 3T3 fibroblasts affecting the relative amounts of CDKs to their inhibitors.

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TPA-MEDIATED GROWTH ARREST OF RHABDOMYOSARCOMA CELLS IN CULTURE ((B.M. Zani*, A. Germani, A. Musarò, A. Mauro*, C. Ciccarelli*, A. Scoglio and M. Molinaro)) Dept. of Experimental Medicine, Univ. L'Aquila, Coppito 2, 67100 L'Aquila, Italy, Inst. of Histology and gen. Embryology Univ. of Rome "La Sapienza" 00161 Rome, Italy.

The human rhabdomyosarcoma derived cell line (RD) undergoes TPA-induced arrest of growth and myogenic differentiation, with no alteration of the expression patterns of the myogenic regulatory genes. Severe alterations of the expression and posttranslational modification of a mutated form of p53 may be involved in the action of TPA (Germani et al, Biochem. Biophys. Res. Commun. 202, 17-24, 1994). There is evidence for connection between the p53 and pRb pathways: p53 induces the expression of p21/WAF1, an inhibitor of CDKs that are responsible of the phosphorylation of pRb, and prevents the release of pRb from E2F leading to growth arrest. pRb can be considered as a downstream effector of p53-induced growth arrest. In line with these evidences we studied the expression of positive and negative regulators of cell cycle progression such as pRb, p21/WAF1, cyclin D1, CDK4 in RD cells. The expression of pRb mRNA does not change during TPA treatment but the phosphorylation level of the protein drastically decreases beginning from day 2 of TPA treatment and at day 6 pRb is present only as hypophosphorylated form. D type CDKs are shown to phosphorylate pRb and to contribute to R point control. While the expression of cyclin D1 is highly increased there is little effect on the level of CDK4. Furthermore the expression p21/WAF1 is increased by TPA treatment with the same time course of cyclin D1. These data are consistent with a dual role of cyclin D1 in positive and negative regulation of the cell cycle as well as with the reported ability of exogenously expressed Rb to induce cyclin D1 expression. The increase of p21/WAF1 reinforces the evidence that D1 CDK complex can be inactivated by TPA leading to growth inhibition of RD cells in culture. The assembly of D type cyclin and CDKs is regulated by growth factors. In RD cells neutralisation of IGFII by specific antibody induces growth arrest and hypophosphorylation of Rb of same extent as TPA, suggesting that TPA induces growth arrest interfering with autocrine growth factor loop.

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INHIBITION OF CELL CYCLE PROGRESSION AT G1/S BY THE CDC42HS-P38/RK SIGNALING PATHWAY. ((A. Molnár, A. M. Theodoras, J. R. Woodgett, L. I. Zos and J.M. Kyriakis)) Diabetes Research Laboratory, Massachusetts General Hospital, Boston, USA, ² Mitotix Inc., Cambridge, USA, ³ Ontario Cancer Institute, Toronto, Canada and ⁴ Howard Hughes Medical Institute, Children's Hospital, Boston, USA. (Spon. By J.M. Kyriakis)

Environmental stress and inflammatory cytokines can activate mammalian signal transduction pathways, including the stress activated p38/RK mitogen activated protein (MAP) kinase pathway. A number of the environmental stress signals, such as ultraviolet light, ionizing radiation, inflammatory cytokines have major effect on the cell cycle and ultimately on the fate of the cell. These signals also activate strongly the p38 pathway. In order to assess the role of p38/impk2 MAPkinase in cell cycle progression we microinjected synchronized NIH-3T3 early in G₁ with expression vectors harboring p38-, SAP- and ERK1 kinases as well as p70 S6 kinase. The effect of the expressed proteins on cell cycle progression was evaluated as a single cell based assay by incorporation of the base analog 5- BrdU. Transient expression of p38 MAPkinase had a striking 75% inhibition on the cell cycle progression comparing to cells microinjected with the empty expression plasmid only. P70 S6 kinase had no effect, SAPkinase only slightly decreased, while ERK1 modestly accelerated the G₁/S progression. MKK3 and SEK1 are two mammalian MEKs capable of activating p38 in vitro and in vivo. To evaluate further the significance of cell cycle inhibition by p38, we investigated the effect of MKK3 and SEK1 on cell cycle progression. Microinjection of these MEKs resulted in an inhibition of S phase entry similar that seen for p38. Expression of dominant negative mutant forms of MKK3 or SEK1 could abrogate completely the inhibitory effect of coexpressed p38 on cell cycle progression, while expressing these mutants alone gave no significant effect on S phase entry. Cdc42Hs, a member of the Rho subfamily of small GTP-binding proteins, can activate p38 (and the SAPKs) *in situ* via a mechanism requiring PAKs. To investigate the potential effects of Cdc42Hs on cell cycle arrest, we expressed wild type (wt) Cdc42Hs into synchronized NIH-3T3 cells. Expression of wtCdc42Hs resulted in a striking arrest of the cell cycle at G₁/S, exceeding that seen for p38. Coexpression of dominant interfering (KR) SEK1 and MKK3, while having no effect on Cdc42Hs expression resulted in a partial reversal of the cell cycle inhibitory effects of Cdc42Hs. Taken together, these results suggest that recruitment of the p38 pathway by cytotoxic stress may result in inhibition of cell cycle progression at the G₁/S boundary.

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INHIBITION OF THE GROWTH OF WI-38 FIBROBLASTS BY BENZYL OXYCARBONYL-LEU-LEU-TYR DIAZOMETHYLKETONE: EVIDENCE THAT CLEAVAGE OF p53 BY A CALPAIN-LIKE PROTEASE IS NECESSARY FOR G₁ TO S-PHASE TRANSITION. ((W. Zhang and R.L. Mellgren)) Department of Pharmacology, Medical College of Ohio, Toledo, OH 43614.

The effect of a calpain-selective cell permeant inhibitor, benzylloxycarbonyl Leu-Leu-Tyr diazomethylketone (ZLLY-CHN₂), on the serum-stimulated growth of WI-38 human fibroblasts has been investigated. Only cell permeant protease inhibitors with activity against calpains prevented progression into S-phase. Protein blotting experiments indicated that p53 immunoreactivity increased in late G₁ in cells treated with ZLLY-CHN₂. The content of p21^{WAF1/CIP1} CDK inhibitor also increased, providing a mechanism for the observed failure to enter S-phase. Further studies indicated that p53 could be degraded by a ZLLY-CHN₂-sensitive protease immediately prior to S-phase, but that proteolysis did not occur after this critical time point. Chelation of extracellular Ca²⁺ by addition of EGTA inhibited the p53 degradation. Consistent with proteolysis of p53 in late G₁ phase, μ -calpain immunoreactivity transiently accumulated in cell nuclei at this time. ZLLY-CHN₂ did not appear to increase p53 mRNA in WI-38 cells. Purified μ -calpain required only 1 to 3 μ M Ca²⁺ to proteolyze p53 in WI-38 cell lysates. These results indicate that ZLLY-CHN₂ inhibits progression of WI-38 cells into S-phase by inactivating a calpain-like protease that is responsible for proteolysis of constitutively expressed p53 in late G₁.

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CHARACTERISATION OF THE PHEROMONE INDUCED G1 ARREST IN FISSION YEAST ((B.Stern and P.Nurse.)) ICRF, Cell Cycle Laboratory, 44 Lincoln's Inn Fields, London WC2A 3PX

Yeast pheromones block cell cycle progression in G₁ in order to prepare mating partners for conjugation. We have investigated the mechanism underlying the pheromone induced G₁ arrest in the fission yeast *Schizosaccharomyces pombe*. Pheromone blocks the G₁ function of the cdc2 protein kinase associated with B-type cyclins, whereas activation of an earlier G₁ function, the transcriptional induction of S phase genes by the cdc10/res1 transcription factor, is not affected by pheromone. Down regulation of the cdc2-cyclinB kinase is essential as overexpression of the B-cyclins cdc13 or cig2 severely compromise the pheromone induced G₁ arrest. In addition, lack of rum1, a specific inhibitor of the cdc2-cdc13 kinase activity, bypasses the G₁ arrest. Interestingly, pheromone does not inhibit the cdc2-cyclinB activity in highly elongated cells with the result that these cells fail to arrest in G₁. We suggest that the inhibitory pheromone signal and an activating cell size signal oppose each other in controlling the cdc2-cyclinB kinase activity required for the initiation of S-phase.

AMP

Proteolytic Cleavage of Human p53 by Calpain: a Potential Regulator of Protein Stability

MICHAEL H. G. KUBBUTAT AND KAREN H. VOUSDEN*

ABL Basic Research Program, National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, Maryland 21702

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The p53 tumor suppressor protein is activated in cells in response to DNA damage and prevents the replication of cells sustaining genetic damage by inducing a cell cycle arrest or apoptosis. Activation of p53 is accompanied by stabilization of the protein, resulting in accumulation to high levels within the cell. p53 is normally degraded through the proteasome following ubiquitination, although the mechanisms which regulate this proteolysis in normal cells and how the p53 protein becomes stabilized following DNA damage are not well understood. We show here that p53 can also be a substrate for cleavage by the calcium-activated neutral protease, calpain, and that a preferential site for calpain cleavage exists within the N terminus of the p53 protein. Treatment of cells expressing wild-type p53 with an inhibitor of calpain resulted in the stabilization of the p53 protein. By contrast, *in vitro* or *in vivo* degradation mediated by human papillomavirus E6 protein was unaffected by the calpain inhibitor, indicating that the stabilization did not result from inhibition of the proteasome. These results suggest that calpain cleavage plays a role in regulating p53 stability.

The p53 protein plays an essential role in protection from malignant development, and mutational loss of p53 function is the most frequently detected genetic event in human cancers (31). Although normal development can occur in the absence of p53, loss of p53 function results in elevated rates of tumor development, suggesting that p53 functions primarily as a checkpoint to prevent abnormal cell proliferation (17, 33). p53 activity has been implicated in numerous processes, including regulation of centrosome duplication (21) and spindle checkpoints (14), differentiation (1, 18), senescence (2) and regulation of angiogenesis (15). The best-understood functions of p53 are the activation of a G₁ cell cycle arrest and participation in programmed cell death, or apoptosis (reviewed in reference 5), although many of the p53 activities described could play a role in tumor suppression. The p53 protein functions as a sequence-specific activator of transcription (22, 35, 60), and many cellular genes regulated by p53-responsive promoters have been described. Although there are clearly additional transcriptionally independent activities of p53 which contribute to at least some functions, such as apoptosis (6, 28, 63), in many cells the ability of p53 to function as a transcription factor appears to be an integral part of its ability to suppress tumor cell growth (11, 50, 52, 67).

In most normal cells the p53 protein is expressed at very low levels due to the relatively short half-life of the protein. Accumulation of the p53 protein occurs following DNA damage (43), and activation of p53 is also apparent following other types of stress, such as growth arrest (69), hypoxia (25), or ribonucleotide depletion (40). The DNA damage-induced accumulation of p53 is principally the result of the stabilization of the p53 protein (20, 34), although transcriptional and translational regulation may also occur. Elevation of protein levels appears to be one component of the p53 response, although it is not essential for activation of p53 function (32). It seems likely that the normal p53 response involves both activation of

a latent form of the protein, possibly by phosphorylation or redox regulation, and enhanced stability to increase the amount of p53 in the cell.

Despite significant advances in our understanding of how p53 mediates downstream effects such as cell cycle arrest and apoptosis, relatively little is known about how the stability of the protein is regulated in normal cells and how this stability is enhanced following DNA damage. There is evidence that p53 can be degraded through ubiquitin-dependent proteolysis (42), and cells which are defective for this proteasome-dependent pathway show elevated levels of p53 (9). The role of ubiquitin-mediated degradation is most clearly seen following the interaction of p53 with the E6 protein encoded by the human papillomaviruses (HPV) associated with cervical malignancies. E6, in association with the cell protein E6-AP, can function as a ubiquitin ligase (54) which targets p53 for degradation (56). Cells expressing E6 fail to stabilize p53 in response to DNA damage and cannot activate the normal p53-dependent responses (36). Whether E6 enhances the normal mechanism of p53 degradation or whether a different proteolytic pathway is utilized is not clear, although a cellular homolog for the function of E6 has not been described. Mutant p53 proteins expressed in tumor cells are frequently found at very high levels due to increased stability (68) which appears to result from an alteration in cellular environment rather than resistance to normal degradation by the mutant p53 protein (62). Indeed, several p53 mutants which show enhanced stability in tumor cells are targeted for degradation by E6 as efficiently as the wild-type protein (13, 55).

The nonlysosomal calcium-activated neutral protease, or calpain (EC 3.4.22.17), is present in almost all cells, and at least two isoforms of the enzyme, μ - and m -calpain, which differ mainly in the calcium concentration needed for their activation *in vitro*, exist. Calpain belongs to the family of cysteine proteases and consists of an 80-kDa catalytic subunit and a smaller 30-kDa subunit which is identical in all isoforms. Both subunits can bind calcium, but in addition calpain activity can also be regulated by autoprolysis and the inhibitor protein calpastatin (23), suggesting that, like the proteasome, calpains are part of a regulatory proteolytic system. The exact function of cal-

* Corresponding author. Mailing address: ABL Basic Research Program, NCI-FCRDC, Building 560, Room 22-96, West 7th St., Frederick, MD 21702-1201. Phone: (301) 846-1726. Fax: (301) 846-1666. E-mail: voutsden@ncifcrf.gov.

pain is still obscure, but a role of calpains in platelet aggregation, neuronal long-term potentiation, neutrophil activation (reviewed in reference 10), oocyte maturation (64), and apoptosis (53, 58, 70) has been suggested, and it has been shown that calpain can cleave talin, filamin, fodrin, protein kinase C (10), c-mos (64), and several transcription factors, such as c-jun and c-fos (30, 65).

In this study we demonstrate proteolytic cleavage of p53 by an activity in cell lysates which we identified as calpain. The preferential site for cleavage is at the N terminus of the p53 protein, and inhibition of calpain cleavage correlated with enhanced stability of the p53 protein.

MATERIALS AND METHODS

Cell culture. MCF-7 (breast carcinoma, p53 wild type), C33a (HPV-negative cervical carcinoma, mutant p53), RKO (colon cancer, p53 wild type), and RKO/16E6 cells were maintained in Dulbecco modified Eagle medium–10% fetal calf serum.

Plasmids and in vitro translation. Plasmid pProSp53 encoding human wild-type p53 (47); plasmids encoding mutant p53 protein, pGEM3Z 232Thr, pGEM3Z 245Val (13), pGEM4Z Δ 1 (45), and pRC/CMV 22Gln/23Ser (39); and pGEM HPV16 E6 encoding HPV type (HPV16) E6 protein (12) have been described previously. Plasmids encoding mutants p53 15Ala, p53 15Asp, p53 Δ 28-40, and p53 Δ 41-49 were constructed by site-directed mutagenesis and subcloned into pGEM4Z (44). In vitro translation of human p53 proteins and HPV16 E6 proteins in the presence of [35 S]methionine was performed in rabbit reticulocyte lysate or wheat germ extract as described elsewhere (45).

Inhibitors and purified calpain. Leupeptin, E64, calpain inhibitor I, adenosine-5'-O-C3-thiotriphosphate (ATP γ S), and adenylyl-imidodiphosphate (AMP-PNP) were purchased from Boehringer (Indianapolis, Ind.). Calpastatin peptide and phenylmethylsulfonyl fluoride (PMSF) were from Sigma (St. Louis, Mo.), and the inhibitor of interleukin 1 β -converting enzyme (ICE)-like proteases YVAD-cmk was from Bachem Bioscience (King of Prussia, Pa.). The purified 80-kDa subunit of rabbit m-calpain was obtained from Sigma.

Monoclonal antibodies. Monoclonal antibodies DO-1 (Ab-6), Pab421 (Ab-1), Pab1620 (Ab-5), Pab1801 (Ab-2), and Pab240 (Ab-3) against p53 and anti-c-jun (Ab-1) were purchased from Oncogene Science (Cambridge, Mass.). Monoclonal antibody against the retinoblastoma gene product (Rb) (clone G3-245) was from Pharmingen (San Diego, Calif.). The anti-Raf-1 antibody was kindly provided by D. Morrison (National Cancer Institute, Frederick, Md.).

Cell lysate, nuclear extract, and cytoplasmic fraction. Cell lysates were prepared freshly in ice cold Nonidet P-40 buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.1% Nonidet P-40, 1 mM dithiothreitol, 1 mM PMSF) and rotated for 30 min at 4°C. Cell debris was pelleted by centrifugation (15 min, 13,000 \times g, 4°C), and the supernatant was used in the p53 cleavage assay.

Nuclear extract and cytoplasmic fraction from MCF-7 cells were prepared as previously described (16, 57). To verify the purity of the preparation, aliquots of nuclear extract and cytoplasmic fraction were separated by sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane. The membrane was first probed with a monoclonal antibody against Rb as a nuclear marker protein, stripped, and then reprobed with a monoclonal antibody against Raf-1 as a cytoplasmic marker protein.

Cleavage assay. Either in vitro-translated 35 S-labelled protein (5 μ l) or C33a cell lysate (5 μ l) was mixed on ice with cleavage buffer (25 mM Tris-HCl [pH 7.5], 100 mM NaCl, 3 mM dithiothreitol), and, if required, protease inhibitors or antibodies were added. In cleavage experiments including antibodies (1 μ g/50 μ l), this mixture was preincubated on ice for 30 min. Following addition of 10 μ l of MCF-7 cell lysate or the amount of nuclear extract or cytoplasmic fraction indicated in the figure legends and incubation at room temperature, aliquots were taken at the time points indicated in the figures and subjected to SDS–15% PAGE. Either the gels were dried and evaluated by autoradiography or proteins were transferred onto nitrocellulose membranes by Western blotting and the membranes were probed with anti-p53 antibodies DO-1 and Pab1801. p53 protein was visualized by enhanced chemiluminescence (Amersham, Arlington Heights, Ill.).

Cleavage assays using purified m-calpain (4 U/ml) were carried out in phosphate-buffered saline supplemented with 1 mM CaCl $_2$ at room temperature as described in reference 7. HPV16 E6 protein-mediated in vitro degradation of p53 was performed in the presence of rabbit reticulocyte lysate as previously described (12).

Measurement of p53 half-life. Logarithmically growing cells were preincubated for 1 h in the presence or absence of calpain inhibitor I (16 μ M). Incubation with medium lacking methionine and cysteine for 30 min was followed by a 1-h pulse using medium containing 100 μ Ci of 35 S-labelled methionine and 35 S-labelled cysteine (ProMix; Amersham). Cells were washed in phosphate-buffered saline and incubated in medium containing an excess of cold methionine and cysteine for the times indicated in the figures. Cells which were preincubated

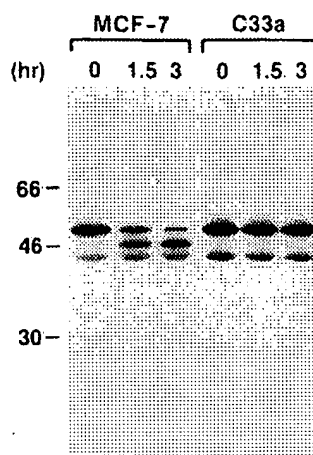


FIG. 1. Cleavage of p53 by MCF-7 lysate. Radioactively labelled in vitro-translated p53 protein was incubated at room temperature with lysates of MCF-7 and C33a cells. Aliquots were taken at the indicated time points, subjected to SDS-PAGE, and evaluated by autoradiography. Sizes are given on the left in kilodaltons.

with calpain inhibitor I were continuously treated with the inhibitor. p53 protein was immunoprecipitated from cell lysates with Pab 421 as previously described (46), separated by SDS-PAGE, and detected by autoradiography.

Immunofluorescence. MCF-7 cells were treated for 16 h with actinomycin D or 3 h with 16 μ M calpain inhibitor I or were left untreated, and immunofluorescence staining was performed as described previously (41) using monoclonal antibody DO-1. DNA was labelled by incubation with 4,6-diamidino-2-phenylindole (DAPI).

RESULTS

Calpain cleaves p53. Previous in vitro studies which have shown efficient ubiquitin-dependent degradation of p53 in rabbit reticulocyte lysate in the presence of HPV E6 have also demonstrated the relative stability of p53 under the same assay conditions in the absence of the E6 protein (12, 56), suggesting that at least one of the components normally regulating p53 proteolysis is missing. In an attempt to reconstitute p53 degradation in vitro, we supplemented p53 protein translated in rabbit reticulocyte lysate with extracts made from MCF-7 cells, a cell line which expresses wild-type p53 which can be stabilized following DNA damage (29). Although we were unable to detect a component in the cell lysate which could substitute for E6 in mediating ubiquitin-dependent proteolysis of the p53 protein, we were able to detect cleavage of the full-length p53 resulting in the generation of a smaller protein of 46 kDa (Fig. 1). This cleavage activity was not detected in C33a cells, a cell line expressing high levels of mutant p53 (66).

Several protease inhibitors were examined to determine the identity of the proteolytic activity in MCF-7 cells. No evidence of ubiquitinated p53 was found, and we were able to confirm that the proteolysis observed was not related to proteasome-associated degradation by demonstrating independence of ATP. Incubation with two ATP inhibitors, AMP-PNP and ATP γ S (Fig. 2A), or preincubation with apyrase (data not shown) failed to inhibit the cleavage of p53 to generate the 46-kDa product. The cleavage was inhibited, however, by EDTA (Fig. 2A), and closer analysis confirmed that the cleavage was calcium dependent. Inhibition of the p53 cleavage by EGTA was clearly relieved by the addition of excess CaCl $_2$ but unaffected by MgCl $_2$ (Fig. 2B). A candidate ATP-independent, calcium-dependent protease is calpain, and this was tested directly by examining inhibition of the p53 cleavage activity by

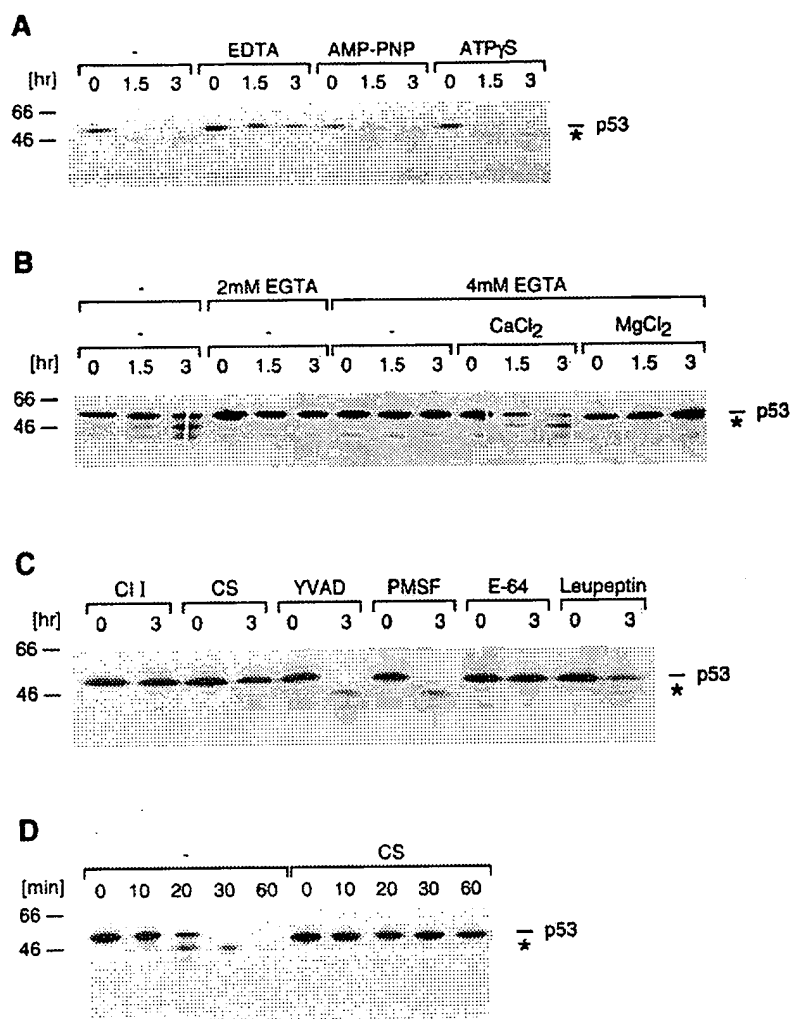


FIG. 2. (A) Effects of EDTA (2 mM), ATP γ S (4 mM), and AMP-PNP (4 mM) on cleavage of p53 by lysate of MCF-7 cells. (B) Calcium (20 mM), but not magnesium (200 mM), can overcome EGTA-mediated inhibition of p53 cleavage by lysate of MCF-7 cells. (C) Effects of various protease inhibitors on cleavage of p53 by lysate of MCF-7 cells. Abbreviations: CI I, calpain inhibitor I (45 μ M); CS, calpastatin peptide (30 μ g/ml); YVAD, YVAD-cmk (100 μ M). Other inhibitors: PMSF, 2 mM; E-64, 50 μ M; leupeptin, 10 μ M. (D) Purified calpain cleaves in vitro-translated p53 in vitro (CS, calpastatin peptide, 100 μ g/ml). Radioactively labelled in vitro-translated p53 protein was incubated at room temperature with lysate of MCF-7 cells or purified calpain (4 U/ml) in the presence or absence (-) of the indicated amount of inhibitor and/or salt. Aliquots were taken at the indicated time points, subjected to SDS-PAGE, and evaluated by autoradiography. The positions of full-length p53 and the cleavage product (*) are indicated. Sizes are given on the left in kilodaltons.

calpain inhibitors (Fig. 2C). Although the cleavage was unaffected by the addition of PMSF (an inhibitor of serine protease) or YVAD-cmk (a specific inhibitor of ICE-like proteases), loss of cleavage activity following the addition of an inhibitor of cysteine proteases (E-64) and more specific inhibitors of calpain (leupeptin, calpain inhibitor I, and calpastatin peptide) confirmed the identity of the protease in the MCF-7 cell extract as calpain. Interestingly, although calpain is expressed in most cells, we were frequently unable to detect the cleavage activity when testing lysates from different cell types (e.g., C33a [Fig. 1]). This did not correlate with p53 levels and is probably due to activity of the endogenous calpain inhibitor, calpastatin, during the preparation of the lysates. Purified rabbit calpain was used in place of the MCF-7 cell lysate and a similar cleavage of p53 was seen (Fig. 2D), although prolonged incubation resulted in further degradation of the primary 46-

kDa cleavage product, an activity which was also sometimes seen following prolonged incubation with MCF-7 cell lysate (see Fig. 3C).

Calpain preferentially cleaves in the N terminus of p53. Cleavage of the p53 protein by calpain to a distinct 46-kDa form suggested the presence of a single preferential cleavage site. In vitro translation of p53 frequently gives rise to two translation products, the full-length protein and a smaller protein of about 42 kDa which is likely to be the product of internal initiation. Since this smaller translation product appeared to be resistant to cleavage in this assay (Fig. 1), we investigated the possibility that a preferred cleavage site for calpain resides within the N terminus of p53. Analysis of a series of p53 mutants showed that point mutants within the DNA binding domain of the protein (232Thr and 245Val), such as those commonly found in human tumors, did not affect

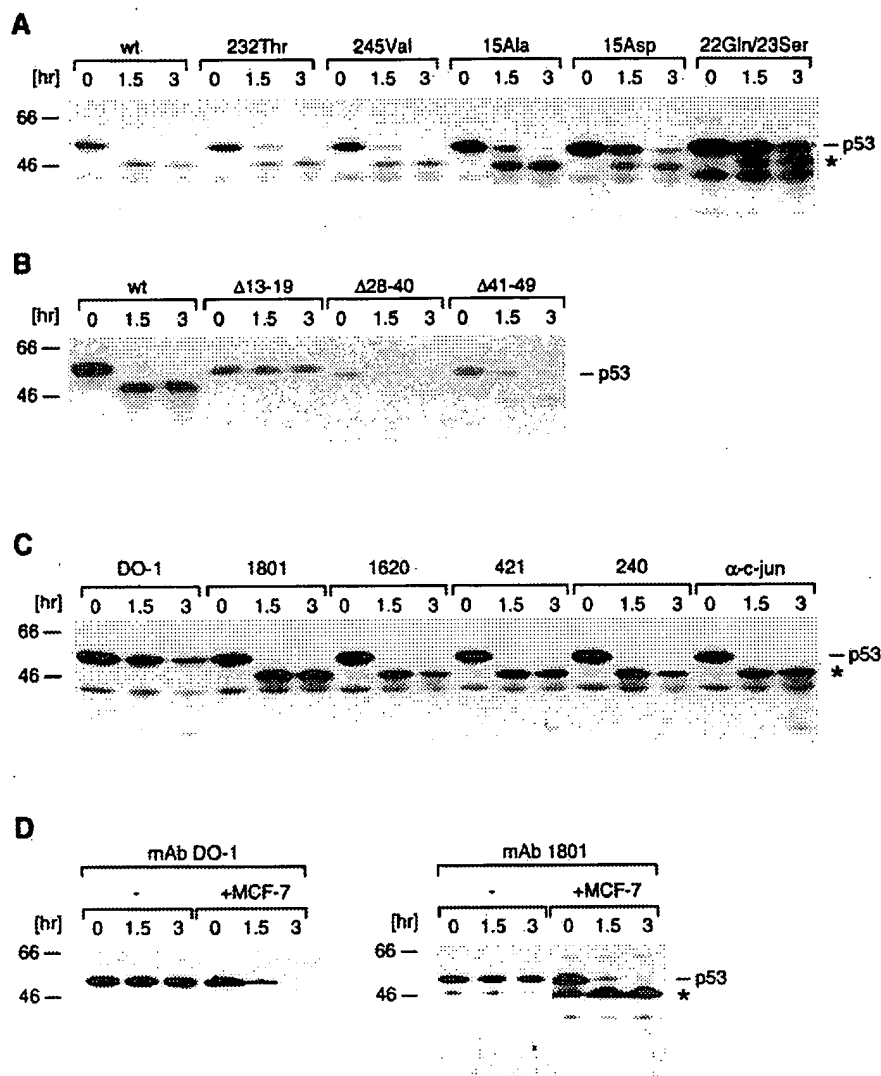


FIG. 3. Analysis of different p53 point (A) and deletion (B) mutants in cleavage assay using lysate of MCF-7 cells (wt, wild type). (C) Effects of different anti-p53 antibodies on cleavage of p53 by MCF-7 cell lysate. In vitro-translated p53 protein was preincubated on ice with monoclonal antibodies (20 μ g/ml), mixed with lysate of MCF-7 cells, and further incubated at room temperature. (D) Western blot analysis for p53 protein in C33a lysate after incubation with lysate from MCF-7 cells. Lysate of C33a cells containing a high level of mutant p53 was incubated at room temperature in the presence or absence (–) of MCF-7 cell lysate. Aliquots taken at different time points were probed for p53 protein by using monoclonal antibodies (mAb) DO-1 and PAb1801. The positions of full-length p53 and the cleavage product (*) are indicated. Sizes are given on the left in kilodaltons.

the sensitivity to calpain cleavage (Fig. 3A). The mutant protein expressed in C33a cells (273Cys) also retained sensitivity to calpain cleavage (Fig. 3D). Mutations altering a phosphorylated serine at amino acid 15, a potential target for double-stranded-DNA-dependent protein kinase, to either alanine or aspartic acid (15Ala and 15Asp) also failed to affect cleavage. However, a p53 protein carrying a double point mutation within the transactivation domain (22Gln/23Ser) showed some resistance to the cleavage. Further analysis of p53 deletion mutants showed that loss of amino acids 13 to 19 (Δ 13-19), encompassing conserved region I, rendered the p53 protein completely resistant to calpain cleavage (Fig. 3B), suggesting that the cleavage or recognition site for the protease must lie within this region of p53. Deletion of residues 28 to 40 or 41 to 49 (Δ 28-40 and Δ 41-49) did not affect calpain cleavage.

In order to confirm that the calpain cleavage site was localized to this region of the p53 protein, the effect of preincubating the p53 protein with various monoclonal antibodies was examined (Fig. 3C). The epitope recognized by the anti-p53 monoclonal antibody DO-1 has been identified as p53 residues 20 to 25 (59), and previous studies have shown that the p53 Δ I mutant is very inefficiently recognized by this antibody (45). Several other antibodies with epitopes distant from the proposed cleavage site were also used, including PAb1801 (recognizing an epitope encompassing residues 46 to 55), PAb240 (recognizing an epitope encompassing residues 211 to 220), PAb421 (recognizing an epitope encompassing residues 371 to 380), and PAb1620 (recognizing a nonlinear, conformational epitope) (38). Binding of an antibody at the DO-1 epitope efficiently blocked the calpain-dependent cleavage of p53, al-

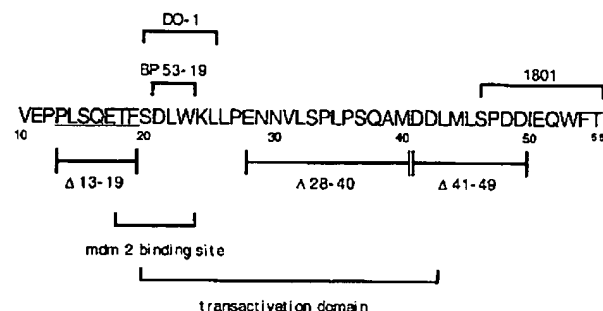


FIG. 4. N-terminal partial amino acid sequence of human p53 protein. Underlined amino acid residues (13 to 19) represent the conserved box I. Marked are the epitopes of antibodies DO-1, BP53-19, and PAb1801; the mdm2 binding site; the transactivation domain; and the deletion mutants used in this study.

though this was unaffected by any of the other p53-specific antibodies or a control antibody which recognizes c-jun (Fig. 3C).

The extent of the N-terminal cleavage was then analyzed by examining the reactivity of the 46-kDa cleavage product with p53-specific monoclonal antibodies. Full-length p53 is recognized by both DO-1 (epitope 20–25) and PAb1801 (epitope 46–55) (Fig. 3D). Although there is some cross-reactivity between PAb1801 and a nonspecific band at around 46 kDa, it is clear that while the appearance of the 46-kDa cleavage product can be detected by PAb1801, only loss of the full-length protein following the calpain cleavage reaction can be detected with DO-1. The inability of DO-1 to interact with the cleavage product is mirrored by a similar lack of reactivity between the cleavage product and the p53 antibody BP53-19 (4), which recognizes a smaller epitope encompassing residues 21 to 23 (59) (data not shown). In summary (Fig. 4), it seems likely that deletion of conserved region I (residues 13 to 19) interferes with calpain recognition and that the cleavage position occurs within the DO-1 epitope (residues 20 to 25). Normal cleavage

of the deletion mutant $\Delta 28-40$ delineates the C-terminal boundary of this site.

Biological relevance of calpain cleavage. Calpain has been localized to the cytoplasm (10) and nucleus of cells (24, 49), and since p53 is predominantly a nuclear protein, we analyzed nuclear and cytoplasmic fractions from MCF-7 cells for p53 cleavage activity (Fig. 5). The purity of each fraction was assessed by detection of a known cytoplasmic protein (Raf-1) and a known nuclear protein (Rb) (Fig. 5B). These analyses revealed a slight nuclear contamination in the cytoplasmic fraction but suggested that the nuclear extract was essentially free from cytoplasmic contamination. Analysis of the cleavage activity in these extracts showed that active calpain could be detected in both cytoplasmic and nuclear extracts, indicating that both p53 and the protease are present in the same cellular compartment (Fig. 5A).

Although clearly evident in the *in vitro* assays, we were unable to detect the 46-kDa p53 cleavage product in lysates from MCF-7 and RKO cells, suggesting either that this proteolytic cleavage does not occur *in vivo* or that the cleavage product is unstable in cells. To test this directly, we examined the p53 levels in MCF-7 cells following incubation with calpain inhibitor I (Fig. 6). Compared to levels in untreated cells (Fig. 6A and B), most of the cells showed elevated p53 levels following treatment with calpain inhibitor I (Fig. 6E and F) similar to those seen following DNA damage induced by actinomycin D (Fig. 6C and D). Increased p53 levels were also seen in these cells following treatment with calpain inhibitor II, although higher concentrations of this inhibitor (100 to 200 μ M) were necessary (data not shown). Since calpain inhibitor I is also a weak inhibitor of ubiquitin-dependent degradation (19, 51), we sought to distinguish between interference with ubiquitin-dependent and calpain-dependent proteolysis by examining the effect of calpain inhibitor I on the ubiquitin-dependent degradation of p53 by E6, a reaction which is clearly calpain independent. RKO cells, which express wild-type p53, and RKO cells expressing HPV16 E6 (36) were examined following treatment with calpain inhibitor I (Fig. 7A), and

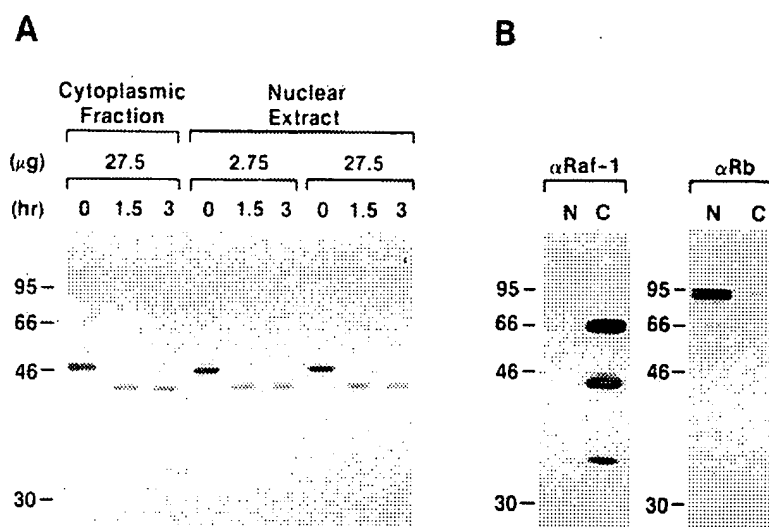


FIG. 5. Proteolytic activity that cleaves p53 protein can be found in nuclear extract and cytoplasmic fraction of MCF-7 cells. *In vitro*-translated p53 protein was incubated with the indicated amounts of nuclear extract and cytoplasmic fraction, and aliquots were subjected to SDS-PAGE and evaluated by autoradiography. (B) Western blot analysis of nuclear extract (N) and cytoplasmic fraction (C) prepared from MCF-7 cells. Samples were probed with antibodies against nuclear Rb protein and cytoplasmic Raf-1 protein. Sizes are given on the left in kilodaltons.

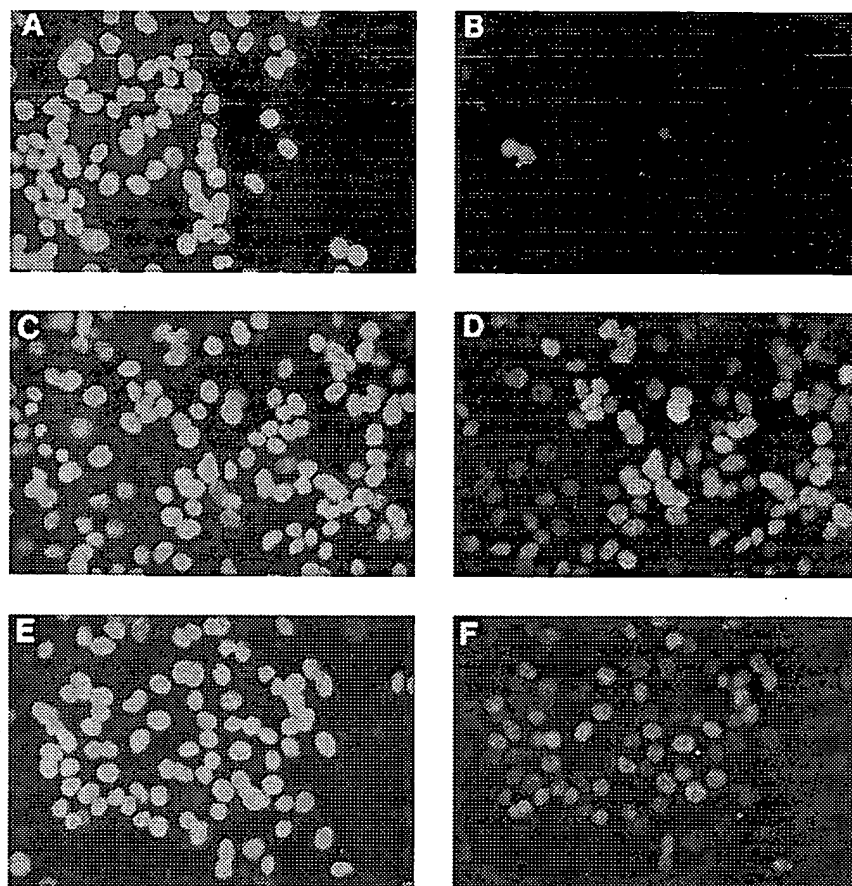


FIG. 6. Levels of p53 protein in untreated (A and B), actinomycin D-treated (C and D), and calpain inhibitor I-treated (E and F) MCF-7 cells. Cells were treated for 16 h with actinomycin D (5 nM) or 3 h with calpain inhibitor I (16 μ M) as indicated, fixed, and probed for p53 protein with antibody DO-1 and fluorescein isothiocyanate-conjugated secondary antibody (B, D, and F). (A, C, and E) DNA was stained with DAPI.

while the p53 levels rose rapidly in RKO cells, barely any stabilization of p53 in the E6-expressing cells could be detected. This is in contrast to the results obtained with efficient proteasome inhibitors, where stabilization of p53 can be clearly detected in both E6-expressing and -nonexpressing cells (42). Degradation of p53 by E6 *in vitro* is inhibited by ATP γ S, which inhibits degradation of ubiquitinated p53 through the proteasomes and results in the accumulation of polyubiquitinated p53 (Fig. 7B). Calpain inhibitor I had no effect on E6-mediated degradation of p53 in these *in vitro* assays (Fig. 7B), however, and no accumulation of ubiquitinated p53 was seen even at inhibitor concentrations more than 10-fold higher than those used to stabilize p53 in MCF-7 and RKO cells. Calpain inhibitor I was also shown to have no effect in the *in vitro* assay under conditions of reduced reticulocyte lysate (which provides the components for ubiquitin-dependent degradation) such that E6-dependent degradation was only weakly evident (data not shown). Taken together, these results indicate that the elevation of p53 levels in cells after treatment with calpain inhibitor I is by virtue of an inhibition of calpain rather than a direct inhibition of the ubiquitin-dependent proteolytic pathway. To verify that inhibition of calpain results in the stabilization of the p53 protein, we carried out pulse-chase experiments with untreated and calpain inhibitor I-treated RKO (Fig. 7C) and MCF-7 (Fig. 7D) cells. Calpain inhibitor I treat-

ment clearly led to an increase of p53 half-life in both cell types.

DISCUSSION

Several mechanisms by which p53 activity may be regulated in cells, including modulation of conformation, phosphorylation, and protein stability, have been described. In this study we show that the p53 protein can be cleaved by calpain *in vitro* to generate an N-terminally truncated protein. Such a cleaved protein would retain many of the major functional regions of p53, including the oligomerization domain and the sequence-specific DNA binding domain, and could be predicted to retain some functions of the wild-type protein or show activity in the negative regulation of the full-length protein. We have, however, been unable to clearly identify the cleavage product in cells, making it more difficult to assess the importance of the calpain cleavage *in vivo*. A p53 mutant which is resistant to calpain cleavage *in vitro* (p53 Δ I) has been shown previously to be more stable when expressed in cells (37, 45), indicating a possible role for the cleavage in the regulation of p53 stability. In support of this suggestion, treatment of cells with calpain inhibitor I was shown to induce a rapid accumulation of p53 protein as a result of enhanced protein stability. This stabilization of p53 is not seen in E6-expressing cells, indicating that

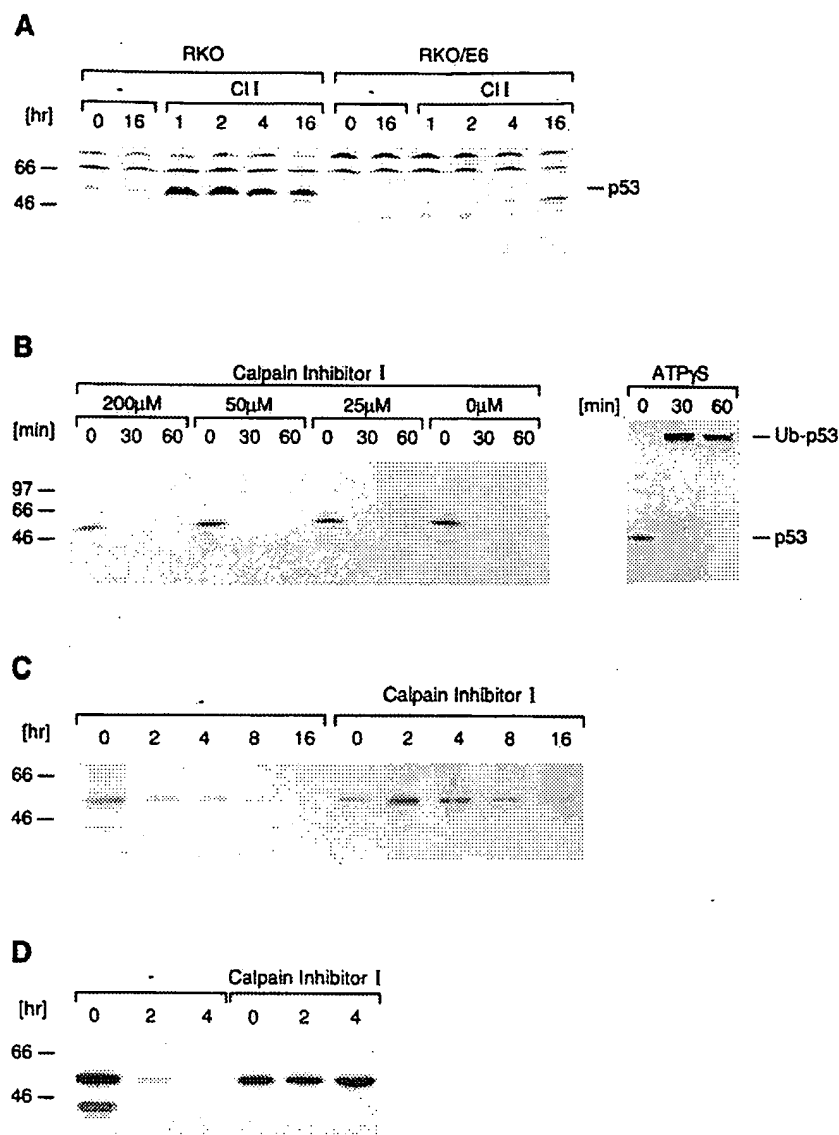


FIG. 7. (A) Western blot analysis of RKO and RKO/E6 cells after treatment with calpain inhibitor I (CI I). Cells were treated with calpain inhibitor I (16 μ M) for the indicated times, and cell lysates were subjected to Western blot analysis using antibody PAb1801. (B) Effects of calpain inhibitor I and ATP γ S on E6-mediated degradation of p53. In vitro-translated p53 was incubated with in vitro-translated E6 protein at room temperature in the presence of rabbit reticulocyte lysate and the indicated amount of calpain inhibitor I or ATP γ S (2 mM). The position of the polyubiquitinated p53 (Ub-p53) is indicated. (C and D) Effect of calpain inhibitor I on the half-life of p53 in RKO (C) and MCF-7 (D) cells. Cells were preincubated with calpain inhibitor I (16 μ M) for 1 h or left untreated (-). After 30 min of incubation in medium lacking methionine and cysteine, cells were labelled with medium containing 100 μ Ci of 35 S-labelled methionine and cysteine and then chased for different periods of time in medium supplemented with excess amounts of cold methionine and cysteine. p53 was immunoprecipitated with monoclonal antibody PAb421. Sizes are given on the left in kilodaltons.

at the concentration used the inhibitor is not significantly affecting proteasome function, consistent with previous studies showing that calpain inhibitor I is a much more potent inhibitor of calpain than of the proteasome (19, 51). Although calpain inhibitor II was somewhat less efficient in the stabilization of p53 in cells, requiring concentrations 5- to 10-fold higher than those of calpain inhibitor I to see an effect, this may reflect a difference in the relative efficiencies of these two inhibitors in vivo. These results are in agreement with a recent study analyzing the role of the proteasome in regulating p53 stability (42), where no stabilization of p53 was seen in cells

treated with lower concentrations of calpain inhibitor II. Dissection of proteolytic activity resulting from proteasome or calpain function using inhibitors is complicated by the observation that in most cases both groups of proteases are affected, although their relative sensitivities differ.

Our inability to detect the p53 cleavage product seen in vitro in cells suggests that this smaller protein is not stable in vivo. It is possible that calpain itself mediates the further degradation of this protein, since we have noted that prolonged incubation with calpain in vitro results in further degradation of the cleavage product. In this case calpain may represent an alternative

pathway to the proteasome for the regulation of p53 stability. Another intriguing possibility is that the N-terminal cleavage of p53 by calpain is a prerequisite for recognition by the ubiquitin pathway and that mutant p53 proteins which are not cleaved by calpain, like p53 Δ I, are resistant to both proteolytic pathways. Although factors governing recognition by the ubiquitin system are not well understood, there is evidence that residues at the N terminus of proteins play a role (3, 61), and it is possible that the new N terminus generated following calpain cleavage represents a more appropriate target for ubiquitin-dependent degradation than that found in the full-length protein.

The suggestion that calpain-dependent cleavage of p53 is necessary for subsequent ubiquitin-dependent degradation raises the interesting possibility that p53 stability following activation by DNA damage is regulated through calpain cleavage. Although we were unable to detect any reduction in the p53 cleavage activity in MCF-7 lysates prepared from DNA-damaged cells, in which the p53 can be shown to accumulate (data not shown), it is possible that DNA damage-induced modifications of the p53 protein inhibit recognition or cleavage by calpain. The localization of the preferred calpain recognition and cleavage site to N-terminal residues presents the potential for regulation of this activity by phosphorylation of residues within this region (48). Although mutation of the serine residue at amino acid position 15 failed to affect calpain cleavage *in vitro*, it is possible that phosphorylation protects the protein from cleavage and that the substitution of aspartic acid in this position cannot adequately mimic phosphorylated serine. Interaction with other cell proteins may also modulate the stability of p53. The mdm2 protein, expression of which is regulated at the transcriptional level by p53, binds directly to the N terminus of the p53 protein and functions to inhibit transcriptional activity and some of the apoptotic functions of p53 (8, 26). The mdm2 binding site on p53 encompasses the region defined in this study as necessary for cleavage by calpain, and the p53 Δ I mutant, which shows resistance to calpain cleavage, no longer interacts with mdm2. Recently, we found that expression of mdm2 leads to the degradation of wild-type p53 *in vivo*, although it has no effect on the stability of the Δ I mutant (27, 37). The mechanism by which this destabilization of p53 is achieved by mdm2 is not known, but it is interesting to speculate that the N-terminal cleavage of p53 by calpain is somehow involved in this process.

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Proteolysis by Calpains: a Possible Contribution to Degradation of p53

MAGALI PARIAT,¹ SERGE CARILLO,¹ MARTA MOLINARI,² CATHERINE SALVAT,¹
LAURENT DEBÜSCHE,³ LAURENT BRACCO,³ JO MILNER,² AND MARC PIECHACZYK^{1*}

Institut de Génétique Moléculaire, UMR 9942, CNRS, 34033 Montpellier Cedex 01,¹ and Centre de Recherche, Rhône-Poulenc-Rorer, 94403 Vitry sur Seine Cedex,³ France, and Yorkshire Cancer Research Campaign p53 Group, Department of Biology, University of York, York YO1 5DD, United Kingdom²

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p53 is a short-lived transcription factor that is frequently mutated in tumor cells. Work by several laboratories has already shown that the ubiquitin-proteasome pathway can largely account for p53 destruction, at least under specific experimental conditions. We report here that, in vitro, wild-type p53 is a sensitive substrate for milli- and microcalpain, which are abundant and ubiquitous cytoplasmic proteases. Degradation was dependent on p53 protein conformation. Mutants of p53 with altered tertiary structure displayed a wide range of susceptibility to calpains, some of them being largely resistant to degradation and others being more sensitive. This result suggests that the different mutants tested here adopt slightly different conformations to which calpains are sensitive but that cannot be discriminated by using monoclonal antibodies such as PAb1620 and PAb240. Inhibition of calpains by using the physiological inhibitor calpastatin leads to an elevation of p53 steady-state levels in cells expressing wild-type p53. Conversely, activation of calpains by calcium ionophore led to a reduction of p53 in mammalian cells, and the effect was blocked by cell-permeant calpain inhibitors. Cotransfection of p53-null cell lines with p53 and calpastatin expression vectors resulted in an increase in p53-dependent transcription activity. Taken together, these data support the idea that calpains may also contribute to the regulation of wild-type p53 protein levels in vivo.

The p53 protein is multifunctional and essential for maintenance of genomic integrity. It can function as a transcription factor (13, 46, 47, 53, 54, 76), but it has also been proposed to play a role in DNA repair (1), in homologous recombination (67), and in the regulation of the translation of its own mRNA (51). p53 can stimulate transcription of certain target genes in vitro and in vivo through the binding to specific DNA sequences termed PREs (p53-responsive elements) (3, 15, 18, 20, 29, 30, 56, 70) found in the promoter regions of genes involved in the control of the cell cycle, such as *Waf1/Cip1* or the cyclin G gene (14, 16), and in DNA repair (52), such as *GADD45* (28). It can also inhibit transcription of a variety of viral and cellular genes devoid of PREs, such as the human genes encoding c-Fos, β -actin (21), proliferating cell nuclear antigen (68), MDR1 (7), and basic fibroblast growth factor (69), probably by interacting with the TATA binding protein. Importantly, tumor-derived p53 mutants are no longer able to bind to PREs and to activate transcription (17, 30, 56, 70). Of paramount importance for its function, the p53 protein can adopt different tertiary structures that can be monitored by using a panel of monoclonal antibodies. Importantly, wild-type p53 protein can both promote and suppress cell proliferation, and these opposing functions correlate to three alternative conformations of the protein (reviewed in references 46 and 47). Moreover, altered function of a number of mutant p53s found in tumors correlates with partially destabilized tertiary structure (47).

Posttranscriptional mechanisms play an important role in p53 gene regulation. In particular, p53 is one of the most unstable proteins found in higher eucaryotes. However, p53

can be dramatically stabilized in response to cues, such as DNA damage, that lead to half-life changes from minutes to hours (13, 23, 33, 34, 54, 61, 76), and a decrease in degradation rate is responsible for the high steady-state level of a number of mutant p53s found in spontaneously occurring tumors. A major route for p53 destruction has recently been shown to involve the ubiquitin-proteasome pathway (9, 38).

Calpains are a family of calcium-dependent intracellular proteases that can be divided into two major groups: the ubiquitous calpains, termed milli- and microcalpains according to the concentration of calcium necessary for revealing their activity in vitro, and tissue-specific calpains (for reviews, see references 11 and 59). Ubiquitous calpains are stimulated by different proteins and phospholipids and are inhibited by a highly specific, high-molecular-weight protein inhibitor named calpastatin. Rather than peptide motifs, they recognize structural determinants, the nature of which remains to be characterized, and they usually cleave their substrates to only a limited extent. Ubiquitous calpains are usually considered exclusively cytoplasmic proteases. This observation is in concordance with the intracellular localization of most of their known or suspected substrates (also see Discussion). Using a series of in vitro degradation assays, we report here that wild-type p53 protein is a substrate for ubiquitous calpains and that degradation is dependent on p53 protein conformation. Modulation of calpain function in vivo, using selected cell lines, affected p53 steady-state levels and transcription functions. This finding indicates that activated calpains may also directly target p53 in living cells and that p53 breakdown may not occur exclusively by the ubiquitin-proteasome pathway.

* Corresponding author. Mailing address: Institut de Génétique Moléculaire, UMR 9942, CNRS, BP 5051, 34033 Montpellier Cedex 01, France. Phone: (33) 467 61 36 68. Fax: (33) 467 04 02 31. E-mail: piechaczyk@igm.cnrs-mop.fr.

MATERIALS AND METHODS

Chemicals. Bovine millicapain (calcium-activated neutral protease), aprotinin, and soybean trypsin inhibitor were from Sigma, and leupeptin was from Boehringer Mannheim. Solid-phase synthesis of the calpastatin peptide inhibitor

(40) was performed on a Milligen 9050 peptide synthesizer, using the fluorenylmethoxycarbonyl group as temporary amino protection. A23187, E64D, calpain inhibitor I, and calpain inhibitor II were from Sigma.

Cells, culture conditions, and preparation of cell extracts. Jurkat and SAOS cells are available from the American Type Cell Collection. H358a (20) and *ts20* (10) cells are kind gifts from J. Roth and H. Ozer, respectively. Cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum at 37°C except *ts20* cells, which were cultured at 32°C. For preparation of S100 cytosolic extracts, Jurkat cells were pelleted by centrifugation, washed twice in phosphate-buffered saline (PBS: 150 mM NaCl, 10 mM sodium phosphate [pH 7]), and then incubated for 5 min on ice in a hypotonic lysis buffer (20 mM HEPES [pH 7.5], 10 mM potassium acetate, 1.5 mM magnesium acetate; 2 ml for 5×10^6 cells) without any detergent. Lysis was completed in a Dounce homogenizer and verified by microscopic examination. Nuclei were removed by low-speed centrifugation ($2,000 \times g$ for 15 min), and supernatants were centrifuged at $100,000 \times g$ for 1 h in a Beckman SW60 rotor. Cytoplasmic extracts (S100; 5 to 12 mg of protein per ml) were then aliquoted and frozen at -80°C until used (4, 6). T3T3 and 3T3tx cells are described in reference 75. For assaying p53 in *ts20* cells, nearly confluent cells were transferred to 39°C for 12 h, and A23187 was added at a concentration of 10 mg/ml in the presence or absence of either E64D, calpain inhibitor I, or calpain inhibitor II at a concentration of 50 μM for the indicated period of times. Cytosolic extracts for immunoblotting analysis were prepared and processed as described elsewhere (48, 75).

In vitro and in vivo expression plasmids. For translation of the human c-Fos protein and of hamster glyceraldehyde-3-phosphate dehydrogenase, we used plasmids FH2 and pSC1 (5), respectively. Most of the wild-type and mutant p53 expression plasmids are described in reference 58. Others were obtained by site-directed mutagenesis of a wild-type p53 cDNA by using standard methods. For cell transfection experiments, we used the following plasmids: (i) RE-CAT, in which the chloramphenicol acetyltransferase (CAT) gene is under the transcriptional control of the CON consensus sequence for p53 (20) placed upstream of a thymidine kinase minimal promoter (8); (ii) SVp53, in which a wild-type human p53 cDNA is placed under the transcriptional control of the simian virus 40 late promoter in the SV2oli vector (64); and (iii) CD136, in which the calpastatin cDNA (26) has been cloned in the *Bam*HI and *Hind*III sites of pCDNAI-neo (InVitrogen).

In vitro transcription and translation. For transcription, plasmids were linearized with appropriate restriction enzymes and subsequently phenol extracted. Transcription using SP6, T3, and T7 RNA polymerases, depending on the expression vector, was performed as specified by the supplier (Promega), as was translation in the rabbit reticulocyte lysate in the presence of [^{35}S]methionine (Amersham). Alternatively, a TNT transcription-translation kit from Promega was used with circular plasmids. Translations were conducted at 37°C unless specified otherwise.

Degradation assays and protein analysis. Degradation experiments were carried out at 37°C in a final volume of 40 μl . For degradation in cytoplasmic extracts, 1 μl of the translation mixture and 35 μl of pure or diluted S100 extract were mixed and prewarmed at 37°C. CaCl_2 (1 mM, final concentration) and, when necessary, protease inhibitors (see below) were then added in a volume of 4 μl at a time taken as time zero. When one component was omitted, the volume was adjusted to 40 μl with PBS. When degradation kinetics analyses were carried out in the presence of pure bovine millicapain, the latter was usually added at a final concentration of 50 $\mu\text{g}/\text{ml}$. For degradation kinetics experiments, aliquots of the reaction mix were sampled at various time points and the reaction was stopped by addition of electrophoresis loading buffer containing 1% sodium dodecyl sulfate. Samples were then electrophoresed through 15% gels as described by Laemmli (34) and electrotransferred onto nitrocellulose for autoradiography. When needed, protease inhibitors were used as follows: calpastatin peptide at 0.5 $\mu\text{g}/\text{ml}$, leupeptin at 5 $\mu\text{g}/\text{ml}$, aprotinin at 200 $\mu\text{g}/\text{ml}$, and soybean trypsin inhibitor at 0.85 $\mu\text{g}/\text{ml}$. Since significant variations of calpain activity are observed from one cell extract to another, susceptibilities to calpains of the different proteins tested here were always compared at 37°C in parallel experiments using the same S100 cytoplasmic extract batch. When accurate comparisons were sought, degradation kinetics experiments were conducted with diluted extracts to slow down degradation rates. The mouse (12) and human recombinant p53 proteins were produced in the baculovirus system and *Escherichia coli*, respectively. Production of human p53 in insect cells was conducted as specified by the manufacturer after cloning of a wild-type p53 cDNA into the pBlueBac III vector (InVitrogen). Extraction from *Spodoptera frugiperda* Sf9 cells nuclei was performed as described in reference 12, and immunopurification was performed as described in reference 37. One microgram of pure recombinant protein was digested in a final volume of 40 μl of a PBS solution containing 1 mM CaCl_2 and various amounts (up to 50 $\mu\text{g}/\text{ml}$) of pure bovine millicapain.

Immunoprecipitations and immunoblotting analysis. Antibodies and conditions for immunoprecipitation of mutant and wild-type p53 have been described previously (41). Immunoblotting experiments were conducted essentially as described elsewhere (48, 75). Fifty micrograms of cell extract was used in the case of MCF7 and *ts20* cells, and the immunodetection of p53 was performed with monoclonal antibody X77 (36) and an enhanced chemiluminescence kit from Dupont. Efficiency of protein transfer onto a nitrocellulose membrane was checked by using Ponceau red staining, and when necessary, immunoblots were

probed with an anti-glyceraldehyde-3-phosphate dehydrogenase antibody for verifying that the same amount of protein was analyzed in each sample.

Cell transfection experiments. H358a and SAOS cells were seeded at a density of 2×10^5 cells per well of six-well culture plates (Nunc). They were transfected 18 h later by using Lipofectin (Gibco/BRL) as specified by the supplier, and CAT assays were performed after a further 36 h of culture according to standard procedures (8), using a Packard Instant Imager Instrument for analysis of thin-layer chromatography plates. Five hundred nanograms of the pRE-CAT reporter plasmid, 10 ng of the SVp53 p53 expression plasmid, when present, and various amounts of PM194 calpastatin expression vector were used per assay. p53 and calpastatin genes were placed under the transcriptional control of the simian virus 40 late promoter and of the cytomegalovirus promoter, respectively. Absence of SVp53 or reduced amounts of PM194 were consequently compensated for by addition of SV2oli and pCDNAI-neo, respectively, to keep constant the concentrations of both promoters in all transfection assays. MCF7 cells, seeded at a density of 10^6 cells per 60-mm-diameter culture dish, were transfected with various amounts of PM194, using Lipofectamine (Gibco/BRL) as specified by the supplier. The concentration of DNA was adjusted to 10 μg per transfection by using pCDNAI-neo. Immunoblotting analysis was carried out 36 h later.

RESULTS

Wild-type human and mouse p53 proteins are substrates for calpains in vitro. The sensitivities of human and mouse p53 to calpains were tested by using a previously described in vitro assay (4, 5; also see Materials and Methods). Briefly, p53 was produced by translation in a rabbit reticulocyte lysate, which is devoid of any detectable calpain activity, and then mixed with an S100 human Jurkat or Daudi cell cytoplasmic extract, which contains the calpain activity, and finally degradation reactions were started by adding calcium for activating calpains. Alternatively, purified bovine millicapain was directly added to the reticulocyte lysate in certain experiments. Translation reactions most often gave rise to a relatively complex pattern of peptides. The longest peptide corresponded to the full-length p53, and the shorter ones resulted from either internal initiation or premature termination. Importantly, the suppressor conformation of the in vitro-translated proteins was confirmed before each degradation experiment by immunoprecipitation using monoclonal antibodies Pab240 and Pab1620 as previously described (41).

Initial degradation experiments were carried out in the presence of 200 μM calcium, which allows the activation of both micro- and millicapains, since the former requires 2 to 75 μM calcium for half-maximum activity and the latter requires 200 to 800 μM (11). Similar results were obtained whether the protein was of human or murine origin. p53 was stable in the reticulocyte lysate for at least 2 h. When both calcium and the S100 cytoplasmic extract were added, p53 decayed rapidly (Fig. 1). Proteolysis was reproducibly two- to fivefold slower than that of the c-Fos proto-oncoprotein, which was processed in parallel and which is one of the most susceptible substrates identified for calpains (4, 5) (Fig. 1). Degradation was specific, since no decay of hamster glyceraldehyde-3-phosphate dehydrogenase and mouse dihydrofolate reductase was detected in experiments conducted in parallel (Fig. 1, lower panels). Degradation was limited, since a number of proteolytic products accumulated in the assay, in keeping with the notion that calpains usually degrade their substrates to a limited extent (11). The proteolytic product pattern was very simple in the case of the mouse protein, with the accumulation of two major bands of 33 and 41 kDa (also see below), and was complex in the case of human p53. In the latter case, the complexity is partly due to the complex pattern of translation products in the reticulocyte lysate that complicates the analysis. Typical degradation kinetics are presented in Fig. 1.

As a next step, we verified that calpains, but not another, unidentified calcium-dependent protease(s), were responsible for the cleavage of p53. Calpain activity (with no discrimination between micro- and millicapains) can be identified by

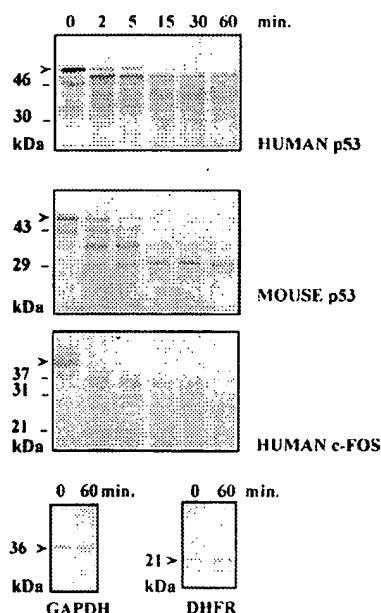


FIG. 1. Calcium-induced degradation of human and mouse wild-type p53 in Jurkat cell S100 cytoplasmic extract. Proteins were translated in a reticulocyte lysate, and degradation kinetics experiments were performed with Jurkat cell S100 cytoplasmic extract (10 mg/ml) in the presence of 200 μ M CaCl_2 as described in Materials and Methods. Time zero corresponds to the addition of calcium for activating calpains. Full-length proteins are indicated by arrowheads. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DHFR, dihydrofolate reductase.

using a series of protease inhibitors (for a review, see reference 73). On one hand, calpains are inhibited by calcium chelators such as EGTA, by cysteine protease inhibitors such as leupeptin, and most specifically by the highly specific endogenous inhibitor calpastatin or by a 27-mer synthetic peptide derived from it (called hereafter calpastatin peptide) (39). Calpains are not inhibited by soybean trypsin inhibitor and the serine protease inhibitor aprotinin. Calcium-dependent degradation of p53 was insensitive to soybean trypsin inhibitor and aprotinin but totally inhibited by EGTA, leupeptin, and the calpastatin peptide (Fig. 2A), thus demonstrating the involvement of calpains. We next showed that both micro- and millicapain can induce p53 cleavage *in vitro*. First, cleavage of both mouse and human p53 was obtained in the presence of 50 μ M calcium only (that is, a condition under which millicapain is not activated) (Fig. 2B). The degree of degradation of p53 was, however, less at 50 μ M than at higher calcium concentrations. This is most likely due to exhaustion of microcalpain activity under the experimental conditions used and not to differences in cleavage specificity between micro- and millicapain, since a p53 protein added after 30 min of incubation was not degraded upon further incubation (not shown). Second, addition of purified bovine millicapain directly to the reticulocyte lysate in the presence of 1 mM calcium caused rapid p53 decay (Fig. 2C). Finally, we demonstrated that p53 is itself a substrate for calpains in S100 cytoplasmic extracts rather than for another protease activated (directly or indirectly) through proteolysis by calpains. To this end, we used the following two-step degradation assay as follows: (i) p53 was first incubated for 30 min in Jurkat S100 extract in the presence of calcium, i.e., a time sufficient for activation of a second possible protease and degradation of the input p53 (Fig. 3A, lane b); (ii) fresh p53 was

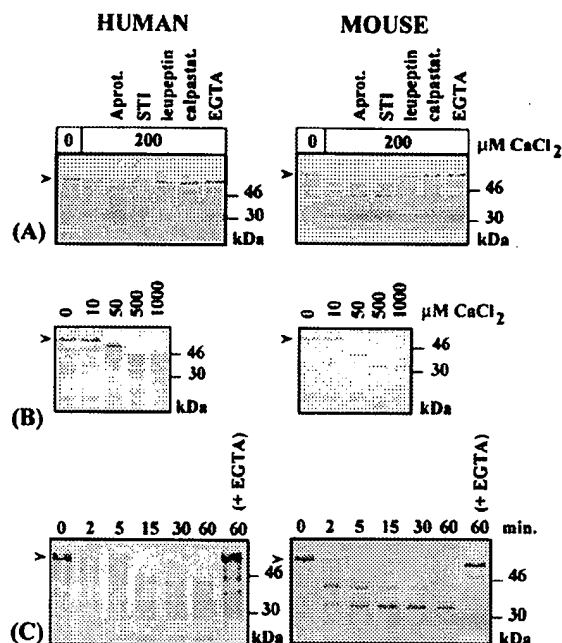


FIG. 2. Both micro- and millicapain can trigger p53 degradation in cell extracts. (A) Action of protease inhibitors on calcium-activated degradation of p53. Degradation experiments were carried out as described for Fig. 1. Reactions were performed in the presence of 200 μ M CaCl_2 for 1 h. Aprotinin (Aprot.), soybean trypsin inhibitor (STI), and leupeptin were added as indicated in Materials and Methods. The calpastatin peptide (calpastat.) and EGTA were added at concentrations of 0.5 mg/ml and 1 mM, respectively. (B) Dependence on calcium. Degradation reactions were performed as for panel A in the presence of various concentrations of CaCl_2 . (C) Effect of millicapain addition to the reticulocyte lysate. Degradation conditions were the same as for panels A and B except that the cytoplasmic extract was replaced by a solution of PBS containing 50 μ g of pure bovine millicapain per ml. CaCl_2 was added at a concentration of 1 mM.

then added to the reaction, and incubation was continued for another 30 min under conditions that are permissive (i.e., in the presence of calcium) or nonpermissive (i.e., in the presence of EGTA or of the calpastatin peptide) for calpains. In the presence of calcium alone, p53 decayed rapidly, indicating that the p53-specific protease is not exhausted after 30 min of incubation (Fig. 3A, lane c). In contrast, calcium chelation (Fig. 3A, lane d) or, more significantly, the calpastatin peptide (Fig. 3, lane e) blocked degradation, thus implicating calpains as responsible for p53 proteolysis in the assay.

The degradation of pure recombinant wild-type mouse and human p53 by purified bovine millicapain *in vitro* in the presence of calcium provided conclusive evidence that p53 is a substrate for calpains. At a high calpain concentration, a 33-kDa fragment prominently accumulated in the case of the mouse p53, in agreement with the size of the shorter proteolytic product generated in S100 cytoplasmic extracts. A more complex pattern of bands was again observed in the case of the human protein (Fig. 3B). It is of note that this experiment indicates that there is no absolute dependence on any cytoplasmic cofactor for p53 degradation by calpains.

In vitro-translated mouse p53 usually yields a simpler proteolytic product pattern than the human protein, as only two major peptides (peptide 1 [41 kDa] and peptide 2 [33 kDa]) are observed, thus making it a better candidate for the mapping of calpain cleavage sites. The peptide fragments were mapped by immunoprecipitation using monoclonal antibodies PAb248

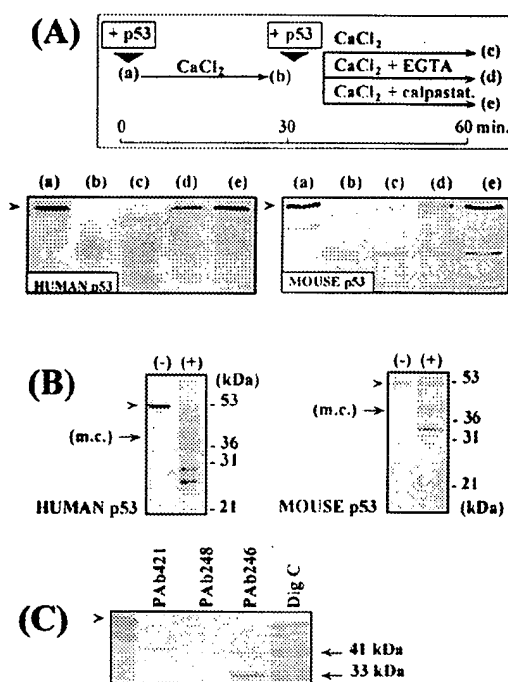


FIG. 3. p53 is a substrate for calpains. (A) Two-step degradation assay. The top panel outlines the experimental protocol. (a) p53 was incubated for 30 min in the presence of 200 μM CaCl₂ in Jurkat S100 cytoplasmic extract. (b) The reaction mixture was split into three aliquots, and the reaction was continued for another 30 min in the presence of CaCl₂ (c), CaCl₂ plus EGTA (1 mM) (d), or CaCl₂ plus the calpastatin peptide (0.5 mg/ml) (e). The full-length p53 is indicated by an arrowhead to the left of each gel. (B) Degradation of recombinant human and mouse p53 by millicalpain. One microgram of either recombinant human or mouse p53 (–) was incubated in the presence of various concentrations of pure bovine millicalpain in the presence of 1 mM CaCl₂ for 1 h. Endpoint degradations (+), corresponding to 50 μg of calpain per ml, are presented. (m.c.) indicates an autoproteolytic product of millicalpain which is generated during the experiment and which can be visualized in degradation assays conducted with only millicalpain (not shown). (C) Immunotyping of mouse proteolytic products. Mouse p53 (indicated by an arrowhead) was degraded to only a limited extent (lane DigC) in a Jurkat S100 extract in the presence of 200 μM CaCl₂. Immunoprecipitations were performed as described elsewhere (41), using monoclonal antibodies PAb421, PAb248, and PAb246. DigC corresponds to a polyclonal antiserum directed against the whole p53 protein. Tenfold more protein was loaded on the electrophoresis gel in the case of control p53 and of DigC-immunoprecipitated p53.

(amino terminus), PAb421 (carboxyl terminus), and PAb246 (central core domain). Both peptides were immunoreactive for PAb246, but none of them was positive for PAb248 and only peptide 1 reacted with PAb421 (Fig. 3C). This result indicates that calpains cleave on both sides of the p53 central core domain responsible for DNA binding (2, 55). Reactivity with PAb246 indicates that the central core domain (peptide 2) retained conformational integrity. Peptide 2 appeared later than peptide 1 in degradation kinetics experiments (Fig. 1 and 2), suggesting that the N and C termini are sequentially removed, with the N terminus being cleaved first.

p53 sensitivity to calpains is determined primarily by the tertiary structure and not by the oligomerization state. Monomers of p53 assemble into dimers and higher-molecular-weight oligomers with high affinity via the oligomerization domain residing at the C terminus of the protein. Human and mouse p53 expression vectors linearized at unique *Ssp*I and *Stu*I restriction enzyme sites, respectively, were used to produce monomeric proteins truncated within the oligomerization do-

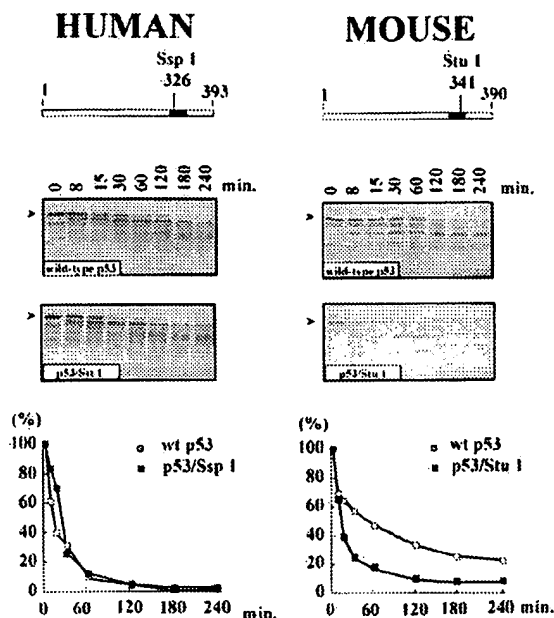


FIG. 4. Degradation of monomeric and oligomeric p53 by calpains. For production of truncated p53, expression vectors for human and mouse p53 were linearized at the *Ssp*I and *Stu*I restriction sites, respectively, before translation in the reticulocyte lysate. Degradation kinetics assays were conducted by using a diluted Jurkat S100 extract (1 mg/ml) in the presence of 200 μM CaCl₂ to allow a more accurate comparison of full-length and truncated proteins. Densitometer analysis of autoradiographs was performed with the Intelligent Quantifier system from Bio Image System. The black boxes on the schemes indicate the localization of p53 oligomerization domains, whereas the numerals indicate amino acid positions. wt, wild type.

main (49), which were tested for their sensitivity to calpains (Fig. 4). Truncated monomeric mouse and human p53 displayed a sensitivity close to that of full-length proteins, thus showing that an intact quaternary structure is not a prerequisite for cleavage.

As calpains recognize structural, rather than sequence, motifs, it was important to test the possibility that the various conformations of p53 are unequally sensitive to calpains. First, we showed that heat-denatured (65°C for 5 min) human and murine p53 are resistant to cleavage by calpains (Fig. 5A). The possibility of insolubilization upon heating that would have rendered p53 resistant to calpains was ruled out since under the same conditions, p53 retained its sensitivity to trypsin (not shown). We then used p53 mutants known to be temperature sensitive for conformation to examine whether conformation affects susceptibility to calpain degradation. Wild-type and mutant conformations of p53 are recognized by monoclonal antibodies PAb1620 and PAb240, respectively. The mouse A135V (45) and human A138V (73) p53 mutants were less susceptible to calpains in the PAb1620⁰/PAb240⁺ conformation than in the PAb1620⁺/PAb240⁰ conformation (Fig. 5B and C). The two mutant proteins, however, behave slightly differently in degradation kinetic experiments. The mouse A135V mutant can quantitatively adopt the PAb1620⁺/PAb240⁰ conformation when translated at 27°C and the PAb1620⁰/PAb240⁺ conformation when translated at 39°C. In contrast, A138V is totally in the PAb1620⁰/PAb240⁺ conformation at 39°C but displays a half-half phenotype when translated at 27°C (not shown). For the sake of homogeneity in our degradation experiments, sensitivity to calpains was tested at 37°C. This was possible be-

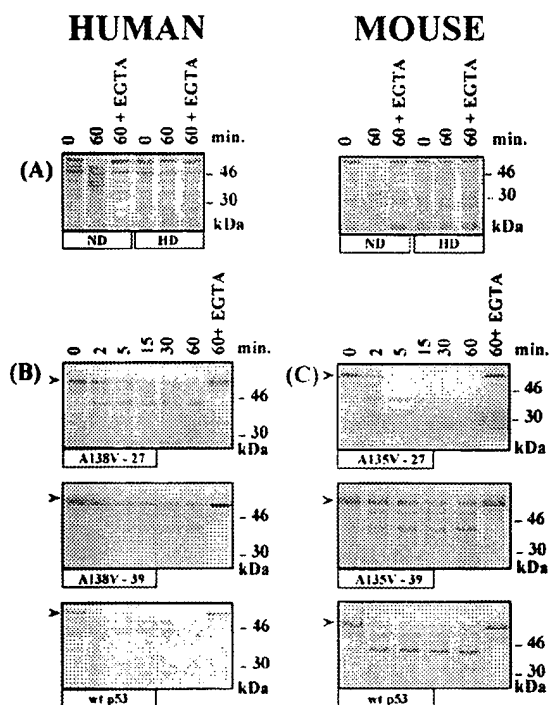


FIG. 5. Influence of p53 tertiary structure upon proteolysis by calpains. (A) Degradation of heat-denatured p53. Human and mouse p53 were produced in the reticulocyte lysate and heated at 65°C for 5 min. The sensitivities to calpains of non-denatured (ND) and heat-denatured (HD) proteins were subsequently compared in a Jurkat cell S100 extract in the presence of 200 μ M CaCl_2 as described for Fig. 1. EGTA was added at a final concentration of 1 mM to block degradation. (B) Susceptibility to calpains of the human A138V p53 mutant translated at 27 and 39°C. A138V was produced in the reticulocyte lysate either at 27°C (A138V-27) or 39°C (A138V-39), and its conformation was tested by immunoprecipitation using antibodies PAb1620 and PAb240 (see text). Degradation kinetic experiments were conducted at 37°C in a Jurkat cell S100 extract (1 mg/ml) in the presence of 200 μ M CaCl_2 in parallel with that of the wild-type (wt) human p53. EGTA was added at a final concentration of 1 mM as a negative control in degradation experiments. (C) Susceptibility to calpains of the mouse A135V p53 mutant translated at 27 and 39°C. The same experiments as in panel B were conducted with the mouse A135V mutant.

cause no detectable amount of protein translated at 39°C undergoes any conformation change when shifted to 37°C, at least for the duration of the experiment (not shown), and the conformation change occurs much more slowly than degradation by calpains when proteins translated at 27°C are shifted to 37°C. Experiments presented in Fig. 5C show that A135V translated at 27°C displays a sensitivity to calpains in S100 Jurkat cell extract comparable to that of the wild-type mouse protein whereas it is a poor substrate for calpains in its PAb1620⁰/PAb240⁺ conformation. Similarly, A138V proved to be a poor substrate for calpains when translated at 39°C and a better one when translated at 27°C (Fig. 5B). Its apparent degradation rate, however, turned out to be lower than that of the wild-type protein when translated at 27°C, with 50% degradation occurring after 5 min of reaction, compared to less than 1 min for wild-type p53. This observation is consistent with (i) the mixed phenotype of A138V at 27°C and (ii) the idea that the PAb1620⁰/PAb240⁺ conformation of this protein is a poor substrate for calpains. Comparable observations were made when pure bovine millicapain was added to *in vitro* translation mixes (not shown).

We next determined whether the p53 protein synthesized in

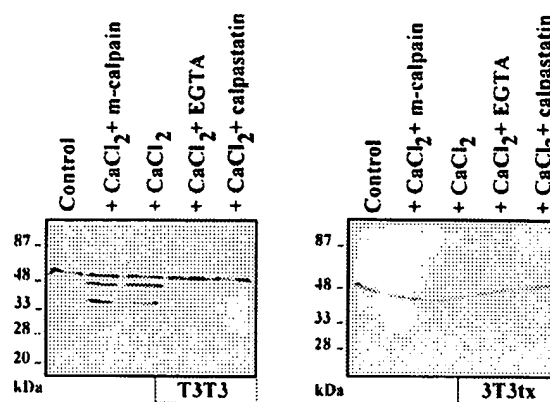


FIG. 6. p53 degradation by calpains in T3T3 and 3T3tx cell cytosolic extracts. Cytosolic extracts were prepared and processed as indicated in Materials and Methods. Degradation experiments were carried out at 37°C for 60 min. Calcium, EGTA, bovine millicapain, and the calpastatin peptide were added at concentrations of 1 mM, 2 mM, 50 μ g/ml, and 0.4 mg/ml, respectively. Immunodetection of p53 was obtained by using a combination of monoclonal antibodies as indicated in Materials and Methods.

vivo was also sensitive to calpains. For this purpose, we used the mouse T3T3 and 3T3tx cell lines, which are derived from BALB/c fibroblasts. Both lines express high amounts of p53 detectable by immunoblotting (48, 75). T3T3 p53 protein harbors two point mutations and adopts a mutant conformation (PAb240⁺) when produced *in vitro* in reticulocyte lysate. Surprisingly, it adopts a wild-type conformation (PAb1620⁺) when expressed *in vivo*. In this case, however, it is predominantly monomeric, with a small proportion being associated with a low-molecular-weight protein (48). 3T3tx p53 also carries two point mutations but is quantitatively found in high-molecular-weight complexes *in vivo* and displays a mutant conformation (PAb240⁺) (48). Cytosolic extracts from both cell lines were prepared, and the sensitivities to calpains of both p53 proteins were tested in parallel experiments (Fig. 6). Addition of calcium triggers the cleavage of T3T3 p53 and the subsequent appearance of two peptides with molecular weights close to those of peptides 1 and 2 (see above). Consistent with a role for calpains, the cleavage is inhibited in the presence of calpastatin or of EGTA. Notably, only half of the protein is broken down, even when exogenous calpain is added to the reaction mixture. The reason for this is not clear, but one possible explanation is that interaction with cell proteins controlling the tertiary and/or the quaternary structure of T3T3 p53 may protect p53 from attack by calpains. 3T3tx p53 was resistant to proteolysis in the presence of calcium even when purified bovine millicapain was added to the extract. This finding rules out the possibility that the absence of cleavage was due to rate-limiting amounts of calpains in this cell context and supports the notion that 3T3tx p53 protein is resistant to calpains because of its mutant conformation.

Natural mutant p53 proteins display different sensitivities to cleavage by calpains. We next compared the susceptibility to calpains of wild-type p53 with those of 10 human p53 mutant proteins found in various types of natural tumors (R175H, M237I, M246V, R248W, R273C, R273H, R273P, T256A, V272M, and D281Y) and of three mouse mutants (C138H, C138P, and H176C [58]). All of the mutants exhibit size fractionation profiles similar to that of wild-type human p53, with peaks equivalent to monomers, dimers, and high-molecular-weight structures, in line with the idea that mutant proteins

TABLE 1. Proteolysis of wild-type and mutant p53 proteins by calpains^a

Protein	Mutant	Sensitivity to calpains
Human	A138V	PS (39°C)
	R175H	PS
	M237I	PS
	M246V	S
	R248W	VS
	R256A	S
	V272M	S
	R273C	VS
	R273H	VS
	R273P	PS
	D281Y	S
Mouse	A135V	PS (39°C)
	C138H	S
	C138P	S
	H176C	S

^aSensitivities to calpains of the human and mouse wild-type proteins were compared to those of p53 mutants in parallel experiments conducted as described for Fig. 7. The flexibility of most mutants has been reported elsewhere (57) (also see text). VS, S, and PS correspond to very sensitive, as sensitive as wild-type p53, and poorly sensitive. See the text and legends to Fig. 5 and 7 for more information.

retain the oligomerization properties of the wild-type protein (57). They are all reactive with antibody PAb240 at 37°C (see Discussion) but not with PAb1620. The sole exception was R248W, which is negative for PAb240 and weakly positive for PAb1620 (57). Three classes of mutants could easily be distinguished (Table 1): (i) mutants that are as sensitive as the wild-type p53, including the human M246V, T256A, V272M, and D281Y and the mouse C138H, C138P, and H176C proteins; (ii) mutants that are more sensitive than wild-type p53, including human R248W, R273C, and R273H proteins; and (iii) mutants with reduced sensitivity to calpains, which include R175H, M237I, and R273P. Typical degradation experiments conducted with Jurkat cell cytosolic extract are presented in Fig. 7. Similar observations were also made when bovine millicalpain was directly added to wild-type and mutant p53-containing reticulocyte lysate (not shown). In the case of mutants showing reduced sensitivity, significant amounts of protein were still detectable after 15 min for R175H and after 1 h for M237I and R273P, while wild-type protein and calpain-sensitive mutants were quantitatively degraded within the 1-min range under the experimental conditions used (Fig. 7B). It is of note that R175H, M237I, and R273P were significantly less resistant than the mouse A135V or the human A138V mutant translated at 39°C (Fig. 5). For analyzing hypersensitive mutants, milder proteolysis conditions were chosen. Whereas 15 to 30 min is necessary for degrading half of the wild-type p53 under the conditions used, R248W, R273H, and R273C decayed quantitatively in less than 2 to 5 min (Fig. 7A). It is noteworthy that the pattern of proteolytic products indicates a more extensive proteolysis of the mutants.

Increased p53-dependent transcription in SAOS and H358a cells transfected with a calpastatin expression vector. Direct assessment of the effect of calpains on p53 turnover in vivo is difficult for several reasons. First, calpains are reputed to be largely, if not exclusively, localized in the cytoplasm. Thus, if p53 is a substrate for calpains in vivo, it should be vulnerable for a short period of time after synthesis since, in principle, it should be protected as soon as it enters the nucleus (also see Discussion). Second, the regulation of calpain activity in vivo is unclear, and no means for exclusive activation of these pro-

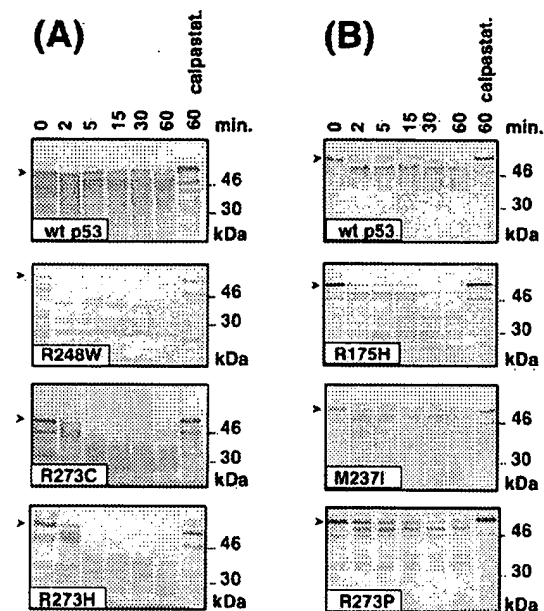


FIG. 7. Sensitivity to calpains of various human p53 mutants. (A) Analysis of mutants with increased sensitivity to calpains. Wild-type and mutant p53 were processed in parallel degradation kinetic experiments as described for Fig. 1 in the presence of 1 mM CaCl_2 . Since overall calpain activities vary from one cell extract to another, a Jurkat cell cytoplasmic extract with low calpain activity was used to show more clearly the differences between wild-type (wt) p53 and the mutants tested in this series of experiment. (B) Analysis of mutants with reduced sensitivity to calpains. Experiments were conducted as for panel A but with a Jurkat cell extract displaying a high calpain activity. calpastat., calpastatin.

teases has ever been described. Third, no cell-permeant protease inhibitor that is strictly specific for calpains is currently available (11, 59, 73). To test the possible involvement of calpains in the regulation of p53 abundance, we initially used an indirect assay based on monitoring p53-dependent transcription in transient transfection experiments. To this aim, the p53-null SAOS (40) and H358a (19) cell lines were transfected with combinations of the following plasmids: (i) a wild-type human p53 expression vector (SVp53) in a limited amount to allow further p53-dependent transcription activity upon p53 stabilization; (ii) a reporter plasmid (RE-CAT) in which transcription of the bacterial CAT gene is under the control of the CON consensus DNA binding motif for p53 (20); and (iii) an expression vector for calpastatin (PM194), the physiological inhibitor of calpains in vivo. No stimulation of the reporter plasmid transcription was observed in the absence of p53 with any amount of calpastatin-expression plasmid. However, co-transfection of increasing amounts of the calpastatin expression vector, together with a constant amount of the p53 expression plasmid, led to a dose-dependent stimulation of p53 transcription activity. This level increased to five- and sevenfold in the cases of H358a and SAOS cells, respectively, under the experimental conditions tested (Fig. 8).

Increased p53 steady-state level in MCF7 cells transfected with a calpastatin expression vector. Since the previous experiments could be interpreted as demonstrating an indirect effect of calpains on the control of p53 transcription activity, it was important to show that modulation of calpain activity could lead to variations in p53 steady-state level. We thus tested the possibility that calpain inhibition could lead to higher accumulation of p53 in living cells. To this aim, MCF7 human breast

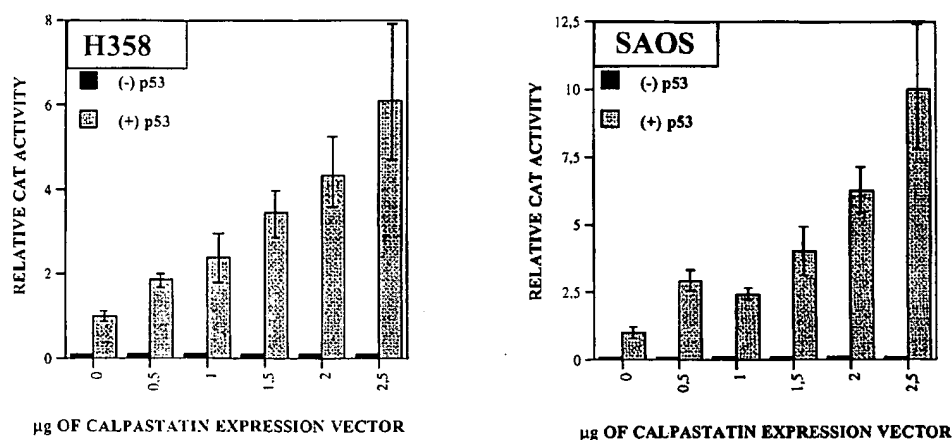


FIG. 8. Stimulation of p53-dependent transcription in H358a and SAOS cells transfected with a calpastatin expression vector. H358a and SAOS cells were transfected as described in Materials and Methods, using the SVp53 p53 expression plasmid or the RE-CAT reporter plasmid expressing the bacterial CAT gene under transcriptional control of the CON consensus binding site for p53 (20) in the presence of various amounts of the PM194 calpastatin expression vector [(+) p53]. Three and two completely independent experiments were conducted with H358a and SAOS cells, respectively. Control transfection experiments with no p53 expression plasmid [(-) p53] were systematically included.

tumor cells, in which wild-type p53 accumulates to easily detectable levels, were transfected with increasing amounts of the PM194 calpastatin expression vector. The anti-p53 monoclonal antibody X77 (36) was used since it also recognizes an unknown protein of 30 kDa which did not vary and was thus useful as an internal reference (u.p. [for unknown protein] in Fig. 9). The results showed a dose-dependent accumulation of

p53 (Fig. 9A) with a maximum threefold stimulation under the conditions used.

Reduced accumulation of p53 in *ts20* cells treated with calcium ionophore. *ts20* cells are thermosensitive for the E1 ubiquitin-activating enzyme of the ubiquitin cycle (9). At the non-permissive temperature (39°C), p53 continuously accumulates over a period of at least 24 h (not shown), because its degradation is essentially ubiquitin dependent in these cells cultured under standard conditions (9). To test the possibility that calpain activation could lead to a reduction in p53 abundance, *ts20* cells cultured at 32°C were transferred at 39°C for 12 h, i.e., a time at which ongoing p53 synthesis provides a situation for proteolytic attack by calpains in the cytoplasm. The calcium ionophore A23187, which activates calpains *in vivo*, was then added, and p53 abundance was subsequently monitored as a function of time by immunoblotting using monoclonal antibody X77. Whereas p53 abundance increased up to 300 to 400% over a 4-h period in the control experiment, only a 50% increase was detected in the presence of calcium ionophore (Fig. 9B).

In addition to activating calpains, calcium ionophore induces a number of other intracellular events that might account for the control of p53 abundance. We therefore repeated the temperature shift experiment, with and without ionophore, this time adding separately protease inhibitors known to penetrate living cells. The inhibitors were E64D, calpain inhibitor I (also called *N*-acetyl-L-leucyl-L-leucyl-L-norleucinal, LLnL, or MG101), and calpain inhibitor II (also called *N*-acetyl-L-leucyl-L-leucyl-L-methioninal or LLM). None is strictly selective for calpains. E64D and calpain inhibitor I also inhibit cysteine proteases such as lysosomal cathepsins, but not the proteasome, whereas calpain inhibitor II also inhibits lysosomal proteases and the proteasome. However, all three inhibit calpains, and it follows that a common effect is likely to involve calpain inhibition. In the presence of ionophore alone, p53 failed to accumulate following a temperature shift to 39°C (Fig. 9C). This result is consistent with those of the previous experiment (Fig. 9B). However, when cells were treated with ionophore plus protease inhibitors, cellular p53 protein increased to levels equal to or greater than those induced in the absence of ionophore (Fig. 9C). Since all three inhibitors had similar effects,

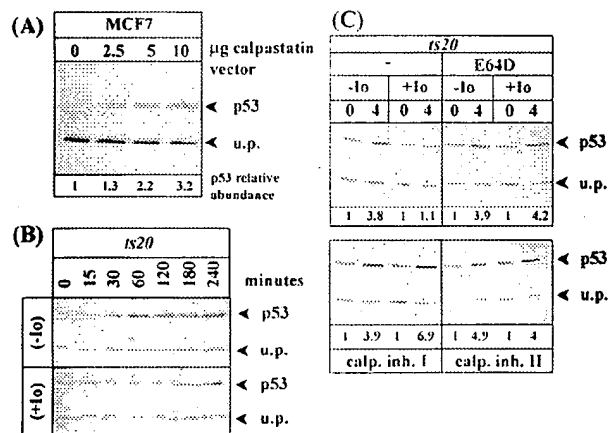


FIG. 9. Variation of p53 steady-state levels in MCF7 and *ts20* cells. (A) Accumulation of p53 in MCF7 cells transfected with PM194. Cell extracts from MCF7 cells transfected with various amounts of the calpastatin expression plasmid were analyzed by immunoblotting using monoclonal antibody X77. u.p. indicates a 30-kDa unknown protein recognized by X77. Relative amounts of p53 were deduced from densitometer analysis of the autoradiogram by using u.p. as an internal invariant reference and are indicated. (B) Reduced accumulation of p53 in *ts20* cells cultured at 39°C in the presence of calcium ionophore (Io). Twelve hours after transfer at 39°C (time zero), *ts20* cells were cultured in the presence or absence of A23187 for various periods of time, and cell extracts were analyzed by immunoblotting using X77. (C) Accumulation of p53 in *ts20* cells cultured at 39°C in the presence of calcium ionophore and calpain inhibitors (calp. inh.). *ts20* cells were processed as for panel B except that calpain inhibitors were added as indicated. Relative amounts of p53 were deduced from densitometer analysis of the autoradiograms, using u.p. as an internal invariant reference, and are indicated. Slight differences in the p53/u.p. signal ratio are visible at times zero. They reflect variations in the accumulation of p53 after 12 h of culture at 39°C.

the increased level of p53 likely resulted from calpain inhibition.

DISCUSSION

p53 conformation and cleavage by calpains. Our results demonstrate that both human and mouse p53 can be cleaved by milli- and microcalpains *in vitro*, and cell studies strongly suggest that p53 may also be a substrate for calpains *in vivo*. While this report was in revision, very similar results were reported by Kubbutat and Vousden for the human protein (33). Cleavage is independent of quaternary structure. However, p53 protein conformation appears crucial for calpain recognition and/or cleavage. Consistent with the notion that calpains do not fully degrade their substrates (11, 59), discrete and stable proteolytic products accumulated in the degradation assays used here for both human and mouse p53. Species-specific patterns were, however, observed. The motifs recognized and cleaved by calpains in their substrate proteins have not yet been clearly identified. They are likely to be of a conformational nature with no strict dependence on specific amino acids at the level of scissile bonds, although amino acid preferences have been reported (11, 59). Thus, it is not clear whether the different degradation patterns are due to slight conformational differences between mouse and human p53 that influence recognition by calpains or whether they are due to amino acid variations at the cleavage sites *per se*. It is important to note that the proteolytic cleavage of p53 by calpains is completely distinct from the proteolytic cleavage induced following interaction of p53 with damaged DNA (50). In the latter case, the cleavage of p53 is observed in calcium-free conditions and also with purified p53, ruling out any involvement of calpains. Nonetheless, there are some similarities between the two cleavage products (of 40 and 35 kDa following interaction with damaged DNA) and a conformationally intact central core domain, reactive with monoclonal antibody PAb246 (Fig. 3C and reference 50). Since some functions of p53 are activated by amino and carboxy truncations, proteolytic cleavage may represent one mechanism regulating the numerous functions of p53.

Because they are sensitive to conformational motifs, calpains can be used as topological probes in protein structure studies. Very interestingly, the different mutants of p53 tested in our survey displayed very different sensitivities to these proteases. Some were as sensitive as the wild-type p53. However, some, such as R248W, R273C, and R273H, were more sensitive, while others, such as the human R175H, M237I, and R273P mutants showed a reduced sensitivity, and another category, which includes the mouse A135V and the human A138V mutants translated at 39°C, showed very low sensitivity. Our data thus support the notion that the different p53 mutants do not adopt a unique conformation but rather adopt a variety of conformations that cannot be discriminated by immunotyping using antibodies PAb240 and PAb1620. It is potentially interesting that the highly sensitive mutants were also more extensively degraded than the wild-type protein in the assay used (Fig. 7A). Whether mutation-linked conformational changes create or expose new cleavage sites to calpains is, however, not clear, since (i) due to the complexity of the pattern of translation products in the case of the human protein, the analysis of degradation products is usually difficult, and (ii) using certain batches of S100 cell extract, we have sometimes observed rather extensive cleavage of wild-type p53 (e.g., Fig. 2). Using a different assay involving a recombinant protein, Bargonetti et al. (2) have shown that (i) p53 possesses multiple cleavage sites for thermolysin on either side of a

protease-resistant central core domain of 27 kDa displaying DNA binding activity and (ii) in the presence of thermolysin, certain mutants, such as R248W and R273H, showed a proteolysis profile similar to that of the wild-type human protein, whereas the core domain was degraded in the case of the V143A and R175H mutants, presumably because of conformation changes induced by the mutations which render cryptic cleavage sites accessible to the protease. Taken with our own observations, this work shows that susceptibility to various proteases may be used for identifying novel variant conformations of mutant p53 that cannot be detected by classical immunotyping.

Contribution of calpains to p53 breakdown *in vivo*? The mechanisms responsible for wild-type and mutant p53 protein breakdown are poorly understood, and multiple catabolic pathways may exist. Moreover, the relative contributions of different pathways may vary according to the cellular context and/or the physiological conditions.

The ubiquitin-proteasome system is responsible for the destruction of numerous intracellular proteins (for reviews, see references 10, 22, 27, 29, and 61). Some evidence is consistent with a role for this system in the degradation of wild-type p53 *in vivo*, since p53 destruction has been shown to require energy (24) and an active E1 ubiquitin-activating enzyme, at least in mouse embryo fibroblasts (9), and inhibition of the proteasome leads to stabilization of p53 and accumulation of ubiquitinated forms of p53 in human fibroblasts (38). One indication that p53 can be degraded by different mechanisms *in vivo* is the fact that E6 proteins from human papillomavirus types 16 and 18 have been shown to accelerate p53 destruction and thereby to favor the development of tumors of the cervix (63). In this case, degradation unambiguously involves tagging of p53 by multiubiquitin chains, due to a cellular protein called E6-AP that interacts with p53 only in the presence of E6 (10, 61, 62, 65). However, whether this corresponds to acceleration of a physiological p53 ubiquitin-dependent degradation pathway or to a deviation toward a more efficient one is not yet clear. It must also be stressed that p53 may also constitute a heterogeneous population of molecules with respect to intracellular proteolytic systems. This is exemplified by the fact that DNA-bound, but not free, p53 is resistant to E6-mediated degradation (50). Interestingly, neither the presence of E6 nor that of oligonucleotides carrying the CON consensus p53 DNA binding site (20) facilitates or inhibits p53 proteolysis by calpains in our *in vitro* degradation assays (results not shown).

We have shown here that the inhibition of calpains in transient transfection experiments leads to a significant elevation of p53-dependent transcription in SAOS and H358a cells. Moreover, elevation of p53 abundance was observed in MCF7 cells transfected with a calpastatin expression vector, and calpain inhibitors allowed p53 accumulation in ts20 cells upon activation of calpains by calcium ionophore (Fig. 8). Since p53 is a sensitive substrate for calpains *in vitro*, these observations raise the interesting possibility that calpains can also contribute to p53 breakdown *in vivo* and thus support the notion that p53 may not exclusively be degraded by the ubiquitin-proteasome pathway. We cannot, however, formally exclude the possibility that calpains regulate p53 steady-state levels by indirect mechanisms and/or modulate p53 transcriptional activity by interfering with intracellular signal transduction cascades. It is of note that the two types of regulation may not be mutually exclusive. If p53 is indeed degraded by calpains *in vivo*, the major issues will be (i) to determine the relative contribution of calpain-mediated destruction of p53 in respect to that of other intracellular proteolytic systems, such as the ubiquitin-proteasome pathway, (ii) to characterize which proteolytic en-

zymes reduce the calpain-generated peptides to amino acids, since neither we (this work and data not shown) nor Kubbutat and Vousden (33) could detect them *in vivo*, and (iii) to determine to what extent resistance to calpains can contribute to increased accumulation of mutant proteins in tumor cells. Concerning the last point, our results suggest that not all p53 mutants would be involved, since only a few of them showed resistance to proteolytic attack by calpains and some of them were even more sensitive. Along this line, it is worth emphasizing that not all p53 mutants are stabilized to the same extent, some mutants even remaining as unstable as the wild-type protein.

It may appear paradoxical that breakdown of a protein reputed to be nuclear might (in part) be achieved by a protease reputed to be exclusively cytoplasmic. It is, however, worth pointing out that (i) p53 has been reported to be cytoplasmic or partially cytoplasmic in various cell contexts, (ii) the transport of nuclear proteins into the nucleus does not always occur immediately after synthesis, (iii) although the possible presence of calpains in the nucleus is controversial, a minor fraction of ubiquitous calpains has been reported to be nuclear in established cell lines (35), (iv) micro- but not millicapain has been shown to be capable of entering the nucleus upon elevation of calcium concentration in digitonin-permeabilized A431 cells (43), (v) several nuclear matrix proteins (42, 44), as well as several transcription factors, have also been shown to be highly susceptible substrates for calpains *in vitro* (4, 5, 66, 72), (vi) apparently, calpain-dependent degradation of p53 could be obtained in a nuclear fraction of MCF7 cells *in vitro* (33), (vii) inhibition of calpains *in vivo* leads to higher c-Fos and c-Jun transcription factor activity in transient transfection assays (reference 29 and our unpublished observations), and (viii) during mitosis, the nuclear envelope gets disrupted, thus allowing interactions between nuclear and cytoplasmic proteins. Taken together, these observations support the notion that nuclear proteins can be substrates for ubiquitous calpains. However, whether proteolysis occurs mainly before (for controlling the abundance of proteins available for transport into the nucleus or the turnover of proteins with blocked nuclear transport, for example) and/or after transport of substrates into the nucleus will have to be elucidated.

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The first two authors contributed equally to this work.

ADDENDUM IN PROOF

Zhang et al. (W. Zhang, Q. Lu, Z.-J. Xie, and R. L. Mellgren, *Oncogene* 14:255–263, 1997) recently reported cell cycle-dependent degradation of p53 by a calpain-like protease in a human fibroblast cell line.

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Apoptosis induction resulting from proteasome inhibition

Kunio SHINOHARA*§, Masanori TOMIOKA*†, Hisako NAKANO*, Shigenobu TONÉ*, Hisashi ITO† and Seiichi KAWASHIMA‡

*Department of Radiation Research and ‡Department of Molecular Biology, Tokyo Metropolitan Institute of Medical Science, Bunkyo-ku, Tokyo 113, Japan, and †Department of Chemistry, Faculty of Science and Engineering, Aoyama-Gakuin University, Setagaya-ku, Tokyo 157, Japan

Proteases are known to be involved in the apoptotic pathway. We report here that benzyloxycarbonyl(Z)-Leu-Leu-leucinal (ZLLLal), a leupeptin analogue, can induce apoptosis in MOLT-4 and L5178Y cells. ZLLLal is a cell-permeant inhibitor of proteasome. Among the protease inhibitors tested, only calpain inhibitor I (acetyl-Leu-Leu-norleucinal) and ZLLLal caused a

marked induction of apoptosis in MOLT-4 cells. In contrast, Z-Leu-leucinal, a specific inhibitor of calpain, did not induce apoptosis. When MOLT-4 cells were incubated in the presence of ZLLLal, p53 accumulated in the cells. These results strongly suggest that inhibition of proteasome induces p53-dependent apoptosis and that proteasome can protect cells from apoptosis.

INTRODUCTION

Apoptosis, a physiologically controlled cell death [1,2], is a complex process which includes the recognition of a signal, signal transduction, and the degradation of cellular DNA. There apparently are a number of factors involved in the apoptosis process. One of the factors may be protease activity, since suppression of protease activity often blocks the development of apoptosis induced by various agents [3–10].

Although little is known about the protease(s) targeted by the inhibitors used, the one best understood is the interleukin-1 β converting enzyme (ICE) homologue, apopain, which is thought to induce apoptosis via the degradation of poly(ADP-ribose) polymerase [11–13]. In addition to apopain, two papers also suggest the involvement of calpain, a calcium-dependent protease, in apoptosis [5,14]. The nature of their involvement is controversial. In one report, inhibitors of calpain blocked apoptosis, showing a positive function of calpain in the induction of apoptosis [5]. In contrast, the other work showed that calpain inhibitors accelerated apoptosis [14], suggesting a negative role. Additional work is necessary to determine the role of calpain in apoptosis, since the so-called calpain inhibitors used in these reports are not specific to calpain; the inhibitors are also effective on proteasome, a multicatalytic protease, and are sometimes used as proteasome inhibitors at the cellular level [15,16].

Proteasome is a unique high-molecular-mass protease complex of 22–31 kDa subunits, and possesses several distinct catalytic activities. Since gene disruption of proteasome subunits is lethal for yeast, proteasome is essential for cell proliferation [17]. In mammalian cells, proteasome is abundantly expressed in malignant leukaemia cells. Its expression increases greatly during blastogenic transformation of normal blood mononuclear cells [18]. These results, together with our finding that the inhibition of proteasome activity induces the differentiation of PC12 cells [19], suggest that proteasome is a positive regulator of cell proliferation, and that its inhibition results in differentiation in some types of cells and possibly in apoptosis in other types, depending on their potentialities.

We have examined the possibility mentioned above, i.e. the involvement of calpain or proteasome in apoptosis, using MOLT-

4 cells. MOLT-4 cells are derived from a human T-cell leukaemia line and die via apoptosis after X-irradiation [20,21], probably due to the induction of p53 [22,23]. In the present work, the effects of various protease inhibitors on the induction of apoptosis were studied. We have found that only inhibitors of proteasome induced apoptosis in MOLT-4 cells, and that this effect was accompanied by an increase in the level of p53.

MATERIALS AND METHODS

Chemicals

Benzyloxycarbonyl(Z)-Leu-Leu-leucinal (ZLLLal) and Z-Leu-leucinal (ZLLal) were synthesized following the method of Ito et al. [24]. Acetyl-Tyr-Val-Ala-Asp-chloromethyl ketone (Ac-YVAD-cmk) and calpain inhibitor I were obtained from Bachem (Bubendorf, Switzerland) and Boehringer Mannheim respectively. All other protease inhibitors were purchased from Sigma. All other reagents were of analytical grade. Chemicals were dissolved in DMSO. Aliquots of the solutions were added to cell cultures. The final concentration of DMSO in the medium was less than 1% (v/v).

Cell line and culture

MOLT-4 cells derived from a human T-cell leukaemia were grown in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum and antibiotics (100 units/ml penicillin and 0.1 mg/ml streptomycin). L5178Y cells derived from mouse lymphocytic leukaemia were cultured in Fischer's medium supplemented with 10% horse serum and antibiotics as for MOLT-4 cells. Cells were maintained at 37°C in a humidified 5% CO₂/95% air atmosphere. The population doubling times were 18 h for MOLT-4 and 8 h for L5178Y cells.

X-irradiation

X-irradiation was performed using a 150 kV X-ray generator unit operating at 5 mA and equipped with an external filter of 0.1 mm Cu and 0.5 mm Al at a dose rate of 0.75–0.78 Gy/min:

Abbreviations used: ZLLLal, benzyloxycarbonyl(Z)-Leu-Leu-leucinal; ZLLal, Z-Leu-leucinal; E64c, (2S,3S)-trans-epoxysuccinyl-leucylamido-3-methyl-butane; E64d, (2S,3S)-trans-epoxysuccinyl-leucylamido-3-methyl-butane ethyl ester; Ac-YVAD-cmk, acetyl-Tyr-Val-Ala-Asp-chloromethyl ketone; TLCK, N- α -tosyl-lysyl chloromethyl ketone; ICE, interleukin-1 β converting enzyme.

§ To whom correspondence should be addressed at, 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113, Japan.

effective energy was 48 keV. Exponentially growing cells (5×10^5 cells/ml) were irradiated in a plastic tissue-culture flask (25 cm^2).

Morphological analysis

Cells were fixed in a solution of methyl alcohol/acetic acid (3:1, v/v). Fixed cells were placed on a glass slide, stained with 1% Giemsa solution, and observed under an optical microscope. Apoptotic cells were scored from morphological changes (nuclear fragmentation) as reported previously [20]. The number of cells counted per sample was more than 1000. The results are shown as an average of three independent experiments.

DNA isolation and agarose-gel electrophoresis

DNA was isolated from 2×10^6 cells using a DNA extraction kit, Sepa Gene[®] (Sanko Junyaku Co. Ltd., Tokyo, Japan). The extracted DNA was treated with RNase A (0.32 mg/ml) for 1 h at 37 °C, and applied to an agarose gel (2%) for electrophoresis. The gel was stained with an ethidium bromide solution (1.0 µg/ml) and observed with a UV light illuminator.

Western blot analysis

Cells were lysed by sonication in an SDS buffer [1% (w/v) SDS/0.04 M Tris/HCl, pH 6.8/7.5% (w/v) glycerol/0.05 M dithiothreitol] containing PMSF (1 mM), leupeptin (10 µg/ml), and pepstatin A (1 µg/ml). Equal amounts of protein (10 µg) were loaded into each lane of a 10% polyacrylamide gel, electrophoresed, and blotted onto a nitrocellulose membrane (Hybond[®]-C extra, Amersham, Little Chalfont, Bucks, U.K.) following the method of Towbin et al. [25]. A monoclonal antibody against human p53 (pAb1801; Novocastra Laboratories, U.K.) was used as the primary antibody. The signal was then developed with the enhanced chemiluminescence Western blot detection system (ECL; Amersham).

RESULTS AND DISCUSSION

To study the involvement of proteases in apoptosis, we examined the effects of a protease inhibitor ZLLLal on X-ray-induced apoptosis in MOLT-4 cells. Table 1 shows the effects of ZLLLal on the induction of apoptosis in X-irradiated MOLT-4 cells. The results indicate that ZLLLal did not inhibit X-ray-induced apoptosis, but that ZLLLal itself induced apoptosis in unirradiated cells in a dose-dependent manner. These results were unexpected in the light of current knowledge of the involvement of proteases in apoptosis (i.e. proteases are involved in the progression of apoptosis). In addition, the effects of ZLLLal and X-rays on the induction of apoptosis were additive,

Table 1 Effects of ZLLLal on X-ray-induced apoptosis in MOLT-4 cells

MOLT-4 cells exposed to 18 Gy of X-rays (+) or unirradiated (–) were cultured at 37 °C for 6 h in RPMI medium (5×10^5 cells/ml) in the absence or presence of 2 or 5 µM ZLLLal. Apoptotic cells were scored from morphological changes (nuclear fragmentation) as reported previously [20]. Results are the means \pm S.E.M. of three independent experiments.

Concentration of ZLLLal (µM)	Percentage of apoptotic cells	
	X-rays (–)	X-rays (+)
0	2.3 \pm 0.8	36.5 \pm 5.5
2	45.8 \pm 2.0	76.0 \pm 2.8
5	51.1 \pm 2.0	76.9 \pm 4.1

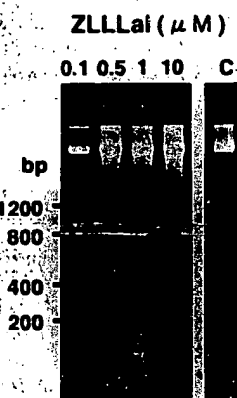
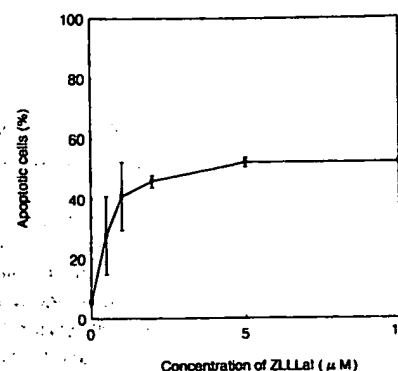


Figure 1 Induction of apoptosis in MOLT-4 cells by ZLLLal

MOLT-4 cells were incubated at 37 °C for 6 h in the presence of various concentrations of ZLLLal. Top: morphological changes. Bottom: DNA fragmentation into nucleosomes and nucleosome multiples (DNA ladder formation). C, control.

suggesting that apoptosis is induced by both agents via a common pathway.

The evidence that the protease inhibitor alone induced apoptosis was confirmed with morphological (Figure 1, top) and biochemical studies (Figure 1, bottom). These results strongly suggest that proteases play an important role in the induction, and also in the suppression, of apoptosis. The induction of apoptosis by ZLLLal was also observed in L5178Y mouse cells (derived from mouse lymphocytic leukaemia cells) when measured morphologically and biochemically (Figure 2). This indicates that the effects of ZLLLal are not MOLT-4 cell specific.

ZLLLal is known to inhibit both calpain [26] and proteasome [27]. We have studied various protease inhibitors (Table 2) to define the role and specificity of ZLLLal in the induction of apoptosis. These included leupeptin for serine proteases and cysteine proteases; (2S,3S)-trans-epoxysuccinyl-leucylamido-3-methyl-butane (E64c) and (2S,3S)-trans-epoxysuccinyl-leucylamido-3-methyl-butane ethyl ester (E64d) for cysteine proteases; N-α-tosyl-lysylchloromethyl ketone (TLCK) for trypsin-like proteases; PMSF for serine proteases; pepstatin A for aspartic proteases; phosphoramidon for metalloproteases; Ac-YVAD-cmk for ICE, a cysteine protease; calpain inhibitor I for calpain and proteasome [15]; and ZLLLal for calpain [28], in addition to ZLLLal. Among the inhibitors examined, only ZLLLal and calpain inhibitor I strongly induced apoptosis (Table 2). We and others have previously reported that ZLLLal and calpain inhibitor I both inhibit protein degradation caused

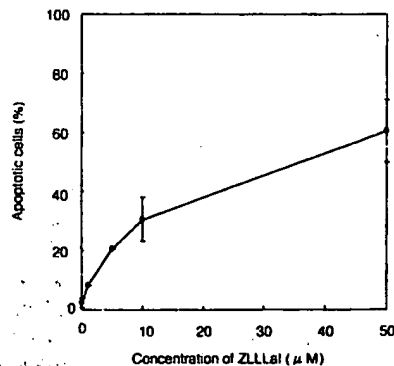


Figure 2 Induction of apoptosis in L5178Y cells by ZLLLal

L5178Y cells were incubated at 37 °C for 6 h in the presence of various concentrations of ZLLLal. Top: morphological changes. Bottom: DNA fragmentation into nucleosomes and nucleosome multiples (DNA ladder formation). C: control.

Table 2 Effects of protease inhibitors on the induction of apoptosis in MOLT-4 cells

Cells were incubated for 6 h in the presence of various protease inhibitors at a 100 μ M concentration. Controls were MOLT-4 cells incubated with 1% (v/v) DMSO instead of an inhibitor. Apoptotic cells were scored from morphological changes (nuclear fragmentation) as reported previously [20]. Results are the means \pm S.E.M. of three independent experiments. The effect of ZLLLal at 1 μ M concentration is also shown for comparison. No morphological changes other than apoptosis were detected in these experiments with the concentrations of inhibitors used.

Protease inhibitors (100 μ M)	Percentage of apoptotic cells
Control	1.9 \pm 0.2
Leupeptin	2.2 \pm 0.5
E64c	1.6 \pm 0.4
E64d	4.3 \pm 2.8
TLCK	8.4 \pm 2.7
PMSF	2.7 \pm 0.8
Pepstatin A	2.2 \pm 0.3
Phosphoramidon	2.2 \pm 0.9
Ac-YVAD-cmk	3.0 \pm 1.4
Calpain inhibitor I	52.2 \pm 6.5
ZLLal	5.4 \pm 1.4
ZLLLal (1 μ M)	42.7 \pm 5.2

by purified proteasomes or calpain [15,27,28]. These results strongly suggest the possibility that inhibition of the proteasome or calpain activity was involved in the induction of apoptosis by



Figure 3 Accumulation of p53 in MOLT-4 cells treated with ZLLLal

Cell lysates from MOLT-4 cells cultured in the presence of 1 μ M ZLLLal or 100 μ M ZLLal were subjected to Western blot analysis. MOLT-4 cells were treated with ZLLLal or ZLLal for various time intervals (h).

these drugs. In addition, ZLLal, which is a strong inhibitor of calpain but a poor one of proteasome [28], did not induce apoptosis (Table 2). Moreover, we have previously reported that ZLLLal induced neuronal differentiation of PC12 cells [19], as did lactacystin (a proteasome-specific inhibitor) [29], suggesting that ZLLLal can also inhibit proteasome activity intracellularly. Taken together, the present observations strongly suggest that ZLLLal and calpain inhibitor I affect cell viability by inhibiting proteasome itself. Some workers have recently argued that the inhibition of calpain-like activity induced the apoptosis observed in their reports [14,30]. They did not consider the possibility of the involvement of proteasome to explain their results. These results also lend support to the idea that cell death caused by ZLLLal or calpain inhibitor I results from the inhibition of proteasome.

It has been demonstrated that p53 is hydrolysed through the ubiquitin-dependent proteolytic pathway catalysed by proteasome [31,32]. We have examined the level of p53 in MOLT-4 cells when incubated in the presence of ZLLLal or ZLLal (Figure 3). The level of p53 increased with time in cells incubated with ZLLLal but not with ZLLal. These results suggest that MOLT-4 cells died through apoptosis due to the accumulation of p53 as a consequence of the inhibition of proteasome. These results are in agreement with evidence that proteasome is involved in the ubiquitin-dependent proteolytic pathway through which p53 is degraded [32], and that overexpression of p53 induces apoptosis [33]. X-irradiated MOLT-4 cells died via apoptosis, which was preceded by an increase in p53 levels (H. Nakano and K. Shinohara, unpublished work). This lends support to the idea that the cause of induction of apoptosis by ZLLLal is an increase in p53, resulting from inhibition of proteasome. This increase in p53 may result primarily from the inhibition of the ubiquitin-dependent proteolytic pathway catalysed by proteasome, which degrades p53, and not from an increase in p53 production, since it has been demonstrated that an increase in p53 protein is not accompanied by an increase in p53 mRNA [34,35]. In conclusion, the present results suggest that inhibition of proteasome induces apoptosis in the cell through the accumulation of p53. Presumably proteasome plays an important role as a regulatory factor influencing physiological levels of p53.

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